A New Mouse model of Peripheral Artery Disease: development, validation and assessment of a potential intervention and a therapeutic target

Thesis submitted by

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Contents

STATEMENT OF SOURCES

I declare that this thesis is my own work and has not be submitted for any other degree or professional qualification. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (Approval numbers A1977, A1978, A2074 and A2321).

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Publications

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- Nsengiyumva, V…., **Omer, S. M**., . . . Golledge, J. (2015). The association of circulating 25-hydroxyvitamin D concentration with peripheral arterial disease: A meta-analysis of observational studies. *Atherosclerosis, 243*(2), 645-651.

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Abstract

Peripheral arterial disease (PAD) affects more than 200 million people worldwide and is caused by occlusions resulting from atherosclerosis in arteries supplying blood to the lower limbs. Treatments for limb ischaemia in PAD is an unmet medical need. Novel treatments are sought to improve limb blood supply and function in PAD patients unlikely to benefit from existing treatments such as surgical revascularisation, structured exercise programs and cilostozol. Currently, the main pre-clinical experimental model employed in PAD research is based on induction of acute hind limb ischemia (HLI) which results in rapid natural recovery of blood supply to ischaemic tissues. There are concerns regarding the ability to translate findings from this mouse model to PAD patients and a clinically relevant mouse model of HLI is lacking. Evidence suggests metformin may be a potential treatment for PAD and angiotensin converting enzyme 2 (ACE2) may play a role in limb ischaemia. This thesis aimed to develop a clinically relevant mouse model of PAD that involved a two-stage induction of HLI and examine the effect of exercise training, metformin and ACE2 deficiency in the two-stage model of HLI.

Two-stage HLI was induced in male Apolipoprotein E (*ApoE*$^{-/-}$) deficient mice by slow onset of severe ischemia over 14 days. This 2-stage HLI model was compared to the acute HLI model and sham controls. Limb blood supply was assessed by Laser Doppler Perfusion Imaging (LDI). Ambulatory ability was assessed using a treadmill test and established scoring scales. Running wheel exercise training was examined in the two-stage mouse model and limb function was assessed by a treadmill exercise test and blood supply was assessed by LDI. Next, the effect of metformin on limb ischaemia was assessed by LDI in the two-stage HLI mouse model. Lastly, the effect of ACE2 deficiency (*ACE2*$^{-/y}$) on limb ischaemia was examined in the two-stage mouse model of HLI.

HLI was significantly more severe in mice receiving the two-stage compared to the acute HLI induction procedure as assessed by LDI ($p=0.013$), and reflected a higher ischemic score ($p=0.003$) and lower average distance travelled on a treadmill exercise test ($p=0.045$). Mice with two-stage HLI receiving exercise training showed significantly greater improvement in their ambulatory ability on a treadmill test than the sedentary control group

(*p=0.003*). However, limb blood supply was comparable between mice receiving exercise compared to controls (*p=0.700*). Mice with two-stage HLI administered metformin had greater blood supply than mice with two-stage HLI receiving vehicle control (*p<0.001*). The two-stage HLI mice receiving metformin had increased adenosine monophosphate kinase alpha (AMPKα) activity (*p=0.041*), endothelial nitric oxide synthase (e-NOS) activity (*p=0.031*), nitric oxide (NO) bioavailability (*p=0.024*), peroxisome proliferator activated receptor 1 alpha (PGC1-α) expression (*p=0.026*) and decreased thioredoxin interacting protein (TXNIP) expression (*p=0.038*) compared to mice with two-stage HLI receiving vehicle. *ACE2*$^{-/y}$ in mice with two-stage HLI had comparable limb ischaemia to ACE2 unmodified control mice with two-stage HLI (*p=0.263*).

In conclusion, this thesis showed that the novel two-stage mouse model of HLI had severe ongoing HLI and functional impairment. Exercise training augmented treadmill walking capacity in the new mouse model which was independent of changes to limb blood supply and mirroring patient response to exercise therapy. Metformin administration improved limb blood supply in the two-stage mouse model of ongoing HLI. Improvement in blood supply was associated with the upregulation of AMPKα activity, increased activation of e-NOS in the ischaemic muscles, increased bioavailability of circulating NO, increased expression of PGC1α and reduced expression of TXNIP. This suggests metformin may have potential to be used as a treatment to improve limb blood supply in PAD patients. *ACE2*$^{-/}$ did not worsen limb blood supply after two-stage HLI induction suggesting ACE2 may not play an important role in limb ischemia. The results do not encourage the pursuit of ACE2 for pharmacological management of PAD in clinical trials and ACE2 is unlikely to be an important target for improving limb blood supply or function in patients with PAD.

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List of Abbreviations

Abbreviation	Name
ABPI	Ankle-brachial pressure index
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACE2$^{-/y}$	Angiotensin converting enzyme 2 deficient
Ad-CMV	Adenoviral cytomegalovirus
ADP	Adenosine di-phosphate
Ad-VEGF	Adenoviral vascular endothelial growth factor
ALDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
Ang 1-2	Angiotensin 1-2
Ang 1-7	Angiotensin 1-7
Ang I	Angiotensin 1
Ang II	Angiotensin II
ANOVA	Analysis of variance
ApoE$^{-/-}$	Apolipoprotein E deficient
ATP	Adenosine tri-phosphate
BH4	Tetrahydrobiopterin
BMC	Bone marrow cells
BM-MNC	Bone marrow mononuclear cells
BP	Blood pressure
C	Celsius
cAMP	Cyclic adenosine monophosphate

CBP	Calf blood pressure
CCR	Chemokine receptor
cGMP	Cyclic guanosine 3’-5’-monophosphate
CLI	Critical limb ischaemia
CV	Cardiovascular
DBP	Diastolic blood pressure
Del-1	Delta 1
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DTT	Di-thiothreitol
EC	Endothelial cell
e-NOS	Endothelial nitric oxide synthase
FA	Femoral artery
FGF	Fibroblast growth factor
g	Gram
GAPDH	Glyceraldehyde-3 phosphate
GM-CSF	Granulocyte monocyte colony-stimulating factor
GTP	Guanosine 5’-triphosphate
HBA_{1c}	Haemoglobin A1c
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor-1
HLI	Hindlimb ischaemia
HRP	Horse radish peroxidase
HSP-90	Heat shock protein-90
IC	Intermittent claudication
IgG	Immunoglobulin gamma
IHC	Immunohistochemistry
IM	Intra-muscular

IQR	Inter-quartile range
kDa	Kilo dalton
Kg	Kilogram
L	Litre
LDI	Laser Doppler imaging
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
LME	Linear mixed-effects
MCP-1	Monocyte c protein-1
mg	Milligram
MI	Myocardial infarction
mL	Millilitre
Mmol	Milli molar
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NEP	Neprilysin
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOD	Non-obese diabetic
NOS	Nitric oxide synthase
NRT	Nicotine replacement therapy
ns	Not significant
NZW	New Zealand white
OCT	Optimum cutting temperature
OD	Optical density
OF	Open field
p	Probability
PAD	Peripheral artery disease

PBS	Phosphate buffered saline
PDE3	Phosphodiesterase 3
PGC1α	Peroxisome proliferator activated receptor
PhD	Doctor of Philosophy
phospho	Phosphorylated
PKA	Protein kinase A
PVDF	Poly-vinylidene di-fluoride
PWT	Peak walking time
Pyr-apelin	Pyroglomulated apelin
qRT-PCR	Quantitative real time polymerase chain reaction
RIPA	radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
s	Seconds
SBP	Systolic blood pressure
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
Ser	Serine
SeV	Sendai virus
SGLT-2	Sodium glucose transporter-2
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween
TcPO2	Transcutaneous oxygen pressure
Thr	Threonine
TXNIP	Thioredoxin interacting protein
V	Volts

VEGF	Vascular endothelial growth factor
x g	Times revolutions per second/ centrifugal force
µL	Microliter
%	Percentage
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to
°	Degree
µg	Microgram
µM	Micro molar

Chapter 1
General Introduction

1.1 Peripheral artery disease, prevalence, risk factors and diagnosis

Peripheral arterial disease (PAD) is an atherosclerotic disease characterised by stenosis and occlusion of the arteries supplying blood to the lower limbs (Golledge 1997, Meneses, Nam et al. 2018). PAD is estimated to affect more than 200 million adults worldwide (Fowkes, Rudan et al. 2013, Shu and Santulli 2018). The prevalence of PAD is 10% in people aged >55 years, nearly 20% in individuals >70 years old and 40% in people aged >80 years (Vavra and Kibbe 2009, Hiramoto, Katz et al. 2014, Krishna, Moxon et al. 2015, Burton, Ademi et al. 2016, Krishna, Omer et al. 2016, Fowkes, Aboyans et al. 2017).

PAD shares similar risk factors to cerebrovascular disease and coronary heart disease including old age, tobacco smoking, hypertension, dyslipidaemia and diabetes mellitus (Norgren, Hiatt et al. 2007, Gerhard-Herman, Gornik et al. 2016). PAD is a marker of systemic atherosclerosis, and PAD patients often have concomitant coronary artery disease or cerebrovascular disease and are at high risk of events such as myocardial infarction (MI), stroke, amputations and death (Askew, Green et al. 2005, Olin, White et al. 2016).

Diagnosis of PAD includes a thorough medical history, physical examination including pulse palpation, ankle brachial pressure index (ABPI) measurement and radiological imaging (Askew, Parmenter et al. 2014). The most common test used to establish the diagnosis of PAD is usually the resting ABPI. The resting ABPI is a simple, non-invasive test involving measuring systolic blood pressures at the arms (brachial arteries) and ankles (dorsalis pedis and posterior tibial arteries) in the supine position by using a Doppler device. The ABPI of each leg is calculated by dividing the higher of the dorsalis pedis pressure or posterior tibial pressure by the higher of the right or left arm blood pressure. Traditionally, ABPI values of 1.00 to 1.30 are considered normal. An ABPI value ≤0.90 is the recognised cut-off for diagnosis of PAD. ABPI values between 0.00 and 0.40 indicate severe PAD, values of 0.41 to 0.90 indicate mild to moderate PAD, values of 0.91 to 0.99 are considered borderline, and values greater than 1.30 indicate non-compressible arteries (Aboyans, Criqui et al. 2012, Gerhard-Herman, Gornik et al. 2016, Olin, White et al. 2016, Kithcart and Beckman 2018). Other non-invasive tools used to diagnose PAD include the post-exercise ABPI, toe-brachial index and duplex ultrasound (Norgren, Hiatt et al. , Gerhard-Herman, Gornik et al. 2016).

1.2 Symptoms of PAD

PAD patients present with a spectrum of symptoms ranging from asymptomatic to limb threatening critical limb ischaemia (CLI) requiring amputation. Asymptomatic PAD patients have faster functional decline, and increased rates of mobility loss compared to people without PAD (McDermott, Greenland et al. 2001, McDermott, Guralnik et al. 2008, McDermott 2015). The two most common presenting symptoms of PAD are intermittent claudication (IC) and CLI. IC presents as debilitating pain of the muscles of the lower extremities that is consistently induced by exercise and consistently relieved by rest. CLI is often preceded by IC and presents as an advanced limb threatening condition of PAD characterised by chronic ($\geq$ 2 weeks) ischaemic rest pain, non-healing wounds, ulcers or gangrene (Gerhard-Herman, Gornik et al. 2016, Kithcart and Beckman 2018).

1.3 Treatments for cardiovascular risk in PAD patients

PAD impairs quality of life in patients and is associated with a greatly increased risk of major cardiovascular (CV) events, amputation and death. Maintaining the independence and physical functions of the elderly preserves their lifestyle and dignity and considerably reduces direct and indirect healthcare costs associated with disability (Norgren, Hiatt et al. 2007, McDermott 2015, Gerhard-Herman, Gornik et al. 2016).

Treatments for PAD patients are centred on reducing the risk of CV and cerebrovascular morbidity and mortality and improving limb haemodynamics and limb function in order to improve mobility, quality of life and prevent amputation (Kithcart and Beckman 2018). Current guidelines recommended for reducing the incidence of CV and cerebrovascular events in PAD patients include smoking cessation, antiplatelet therapy, lowering low-density lipoprotein cholesterol (LDL-C) using statins, blood pressure control and glycaemic control. This is attempted through aggressive lifestyle modifications and medications (Gerhard-Herman, Gornik et al. 2016, Kithcart and Beckman 2018).

1.3.1 Smoking Cessation

Smoking is one of the strongest independent risk factors for PAD and may result in a 7-fold increased risk to develop PAD (Luo, Li et al. 2010). All types of smoking including cannabis, cigar, pipe, smokeless tobacco, and cigarettes predispose to PAD. In addition, passive smoking

is associated with increased risk of developing PAD (T Lu and A Creager 2004, Atturu, Homer-Vanniasinkam et al. 2014, Lu, Mackay et al. 2018).

Smoking is associated with adverse CV events and limb related outcomes including MI, stroke and amputation (Hisamatsu, Miura et al. 2016). Smoking cessation is recommended for all PAD patients. Compared to non-smokers, smokers with PAD have shorter life spans and progress more frequently to CLI and amputation (Uccioli, Meloni et al. 2018). Patients who quit smoking have lower risk of MI and CV mortality and improved amputation-free survival compared with patients who continue smoking (Aronow 2007, Armstrong, Wu et al. 2014, Aboyans, Ricco et al. 2018, Kithcart and Beckman 2018). Strategies to assist patients to quit smoking include active interventions such as behavioural therapy, nicotine receptor partial agonists, antidepressants, and nicotine replacement therapy (NRT). Varenicline, bupropion, and NRT are first line medications, which achieve high-smoking cessation rates and have good safety profiles (Atturu, Homer-Vanniasinkam et al. 2014, Aubin, Luquiens et al. 2014, McDonough 2015).

1.3.2 Antiplatelet/Anticoagulation treatments

Antithrombotic strategies are considered a cornerstone therapy for preventing CV events in patients with PAD (Gutierrez, Mulder et al. 2018). Antiplatelet and anticoagulant agents reduce the risk of thrombus formation, leading to a reduction in serious vascular events in PAD patients (Atturu, Homer-Vanniasinkam et al. 2014). The commonly used antithrombotic medications are cyclooxygenase inhibitors (aspirin), and inhibitors of various platelet surface receptors including P2Y12 (clopidogrel, prasugrel, and ticagrelor), GPIIb/IIIa receptor antagonists (abciximab, tirofiban, and eptifibatide), dipyridamole, phosphodiesterase 3 (PDE3) inhibitor (cilostazol), warfarin, direct thrombin inhibitors (dabigatran and bivalirudin), factor Xa inhibitors (rivaroxaban and apixaban), and heparin (unfractionated, low-molecular-weight heparin) (Atturu, Homer-Vanniasinkam et al. 2014, Gerhard-Herman, Gornik et al. 2016). Aspirin is the most widely used antiplatelet agent and leads to a 25% relative risk reduction of ischaemic stroke, MI, and vascular death (Wong, Chong et al. 2011, Atturu, Homer-Vanniasinkam et al. 2014).

However, studies of aspirin in PAD patients with diabetes mellitus have been entirely negative (Belch, MacCuish et al. 2008, Fowkes, Price et al. 2010). In a meta-analysis of 18 prospective randomised trials comprising 5269 subjects with PAD, aspirin resulted in a 12% reduction of the combined end point of non-fatal MI, non-fatal stroke, and CV death that failed to reach

statistical significance (Berger, Krantz et al. 2009). Clopidogrel has been shown to be more effective than aspirin in reducing adverse CV events (Berger Jeffrey and Hiatt William 2012).

Aspirin and clopidogrel resistance are a growing concern with increased risk of CV events (Guirgis, Thompson et al. 2017). Both metabolic and genetic factors are implicated in drug resistance (Laine, Armero et al. 2013). Prasugrel and ticagrelor have shown to be more efficient with less incidence of resistance (Kastrati 2012). Individualised therapy with platelet function testing has been suggested as a strategy to determine specific anti-thrombotics to reduce CV events in PAD patients (Hess and Hiatt 2018).

1.3.3 Lipid Control

Elevated LDL-C levels are strongly associated with PAD (Murabito, D'Agostino et al. 1997, Gerhard-Herman, Gornik et al. 2016, Aboyans, Ricco et al. 2018, Kithcart and Beckman 2018). Achieving a serum LDL-C of <1.8 mmol/L (<70 mg/dL) or decreasing by approximately 50% if the initial LDL-C level is between 1.8 and 3.5 mmol/L (70 and 135 mg/dL) is recommended for all PAD patients (Piepoli, Hoes et al. 2016). An aggressive reduction in LDL-C with statins reduces all-cause mortality, cardiac death, and increases amputation-free survival in patients with PAD (Aung, Maxwell et al. 2007, Pollak and Kramer 2012, Antoniou, Fisher et al. 2014, Kumbhani, Steg et al. 2014). Even in the most advanced stages of disease, statin therapy is associated with lower 1-year rates of mortality and major CV adverse events (Westin, Armstrong et al. 2014). In patients with coronary artery disease, statins reduce stroke risk (Amarenco, Labreuche et al. 2004, Huang, Li et al. 2013). Ezetimibe is used as an alternative in patients who have contraindications, intolerance or do not respond to statins (Atturu, Homer-Vanniasinkam et al. 2014). Ezetimibe has been shown to significantly reduce levels of LDL-C and has been demonstrated to reduce the rate of CV in high-risk patients (Cannon, Blazing et al. 2015, Hammersley and Signy 2017).

Recently it has been demonstrated that evolocumab, a monoclonal antibody inhibiting the proprotein convertase subtilisin/kexin type 9, reduces LDL-C and the risk of CV events in patients with atherosclerotic disease over statins alone (Sabatine, Giugliano et al. 2017, Bonaca, Nault et al. 2018, Wasserman, Sabatine et al. 2018).

1.3.4 Hypertension

Hypertension is an independent risk factor for PAD and lowering systolic blood pressure (SBP) reduces CV events (Aboyans, Ricco et al. 2018). According to the current guidelines, a target blood pressure <140/90 mmHg is recommended except in patients with diabetes, for whom a diastolic blood pressure (DBP) ≤85 mmHg is considered safe (Gerhard-Herman, Gornik et al. 2016, Aboyans, Ricco et al. 2018, Kithcart and Beckman 2018). In patients with PAD, an appropriate lifestyle and salt intake (<6 g/day) are recommended (Aboyans, Ricco et al. 2018). Diuretics, beta-blockers, calcium antagonists, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers are all used as antihypertensive treatment, as monotherapy or in different combinations according to comorbidities (Atturu, Homer-Vanniasinkam et al. 2014, Aboyans, Ricco et al. 2018). Caution should be taken to avoid an SBP decrease below 110-120 mmHg and lowering DBP below 70mm Hg in patients with CV risk factors may increase the risk of CV events and mortality in older patients, described as a "U" or "J-curve" association (Bavry, Anderson et al. 2010, Gerhard-Herman, Gornik et al. 2016, Aboyans, Ricco et al. 2018, Itoga, Tawfik et al. 2018).

A main concern of lowering SBP is that it could theoretically decrease blood supply to the distal extremities and exacerbate PAD symptoms such as IC and rest pain (Gerhard-Herman, Gornik et al. 2016, Thomas Manapurathe, Krishna et al. 2017). However, a recent meta-analysis has suggested that anti-hypertensive treatment does not worsen but may improve leg ischemia in PAD patients (Thomas Manapurathe, Krishna et al. 2017). Randomised clinical trials of BP targets focused specifically on lower extremity PAD events as the primary outcome of interest are lacking and optimal BP targets for PAD events are unknown (Itoga, Tawfik et al. 2018). Future studies are needed to clarify BP targets for prevention of lower extremity PAD events (Thomas Manapurathe, Krishna et al. 2017, Itoga, Tawfik et al. 2018).

1.3d Glycaemic Control

Diabetes mellitus (DM) increases the risk of developing PAD by 4-fold and leads to increased CV and cerebrovascular event rates, both fatal and non-fatal, in patients with PAD and DM relative to non-diabetic patients with PAD (Thiruvoipati, Kielhorn et al. 2015) (Olin and Sealove 2010). PAD patients with DM are more likely to experience aggressive manifestations of disease, such as worsening lower extremity function and ischaemic ulceration, compared with those with PAD alone (Parvar, Fitridge et al. 2018). The global burden of DM is rising

rapidly and it is predicted that the prevalence of DM in Australia could triple over the next 40 years and the challenge of treating PAD patients with DM will be far greater (Deed, Barlow et al. 2012).

Tight glycaemic control through monitoring of blood glucose and glycated hemoglobin A_{1c} (HbA_{1c}) levels may reduce the incidence of MI, stroke, and vascular death (Norgren, Hiatt et al. 2007). Maintaining HbA1c levels below 7% while avoiding hypoglycaemic episodes is currently recommended (Atturu, Homer-Vanniasinkam et al. 2014, Parvar, Fitridge et al. 2018). Metformin, insulin and sulphonylureas are the most widely used medication to manage hyperglycaemia in DM patients. Other medications include dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-2 analogues and sodium-glucose co-transporter 2 (SGLT-2) inhibitors.

In a recent study, it has been shown that the SGLT-2 inhibitor empaglifozin lowered the risk of CV death by 43% and all-cause mortality by 38% in PAD patients with DM (Verma, Mazer et al. 2018). However, lower limb amputation rates were similar to the placebo group. The SGLT-2 inhibitor canaglifozin has been previously shown to reduce MI, stroke and CV mortality, however canagliflozin was associated with a higher risk of lower limb amputation (Neal, Perkovic et al. 2017). It is unclear whether lower limb amputation risk is higher for specifically canaglifozin treatment or for SGLT-2 inhibitors given that treatment with empaglifozin showed similar lower limb amputation to the placebo group. Therefore, caution is advised for DM patients with PAD for the use of SGLT-2 inhibitors due to the potentially increased risk of lower limb amputation (Everett Brendan and Hiatt William 2016, Parvar, Fitridge et al. 2018).

1.4 Pathophysiology of PAD and treatment of limb symptoms

1.4.1 PAD pathophysiology

The pathophysiological mechanisms underlying the functional impairment and progressive functional decline observed in PAD are complex and incompletely understood (Treat-Jacobson, McDermott et al. 2019). Patients with PAD demonstrate a marked reduction in peak exercise performance and daily ambulatory activity (McDermott, Greenland et al. 2001, Hamburg and Balady 2011, McDermott 2013).

The main underlying cause responsible for the functional limitations in PAD are atherosclerotic obstructions, which affect the supply of oxygen and substrates to metabolically active skeletal

muscle and tissues (Hiatt, Armstrong et al. 2015, Treat-Jacobson, McDermott et al. 2019) (McDermott, Greenland et al. 2001, Hamburg and Balady 2011, McDermott 2013).

In response to arterial occlusions, adaptive revascularisation processes are naturally stimulated to a variable extent in PAD patients (Silvestre, Smadja et al. 2013, Iyer and Annex 2017). This involves a network of specialised endogenous vessels known as collaterals which attempt to restore blood supply (Faber, Zhang et al. 2011). The main adaptive revascularisation processes include arteriogenesis and angiogenesis.

Arteriogenesis involves the rapid transformation of pre-existing collateral vessels and arterioles into functional collateral arteries so that they can deliver more blood to the limb (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). Three major mechanisms are recognised to participate in arteriogenesis: shear stress, inflammation and recruitment of bone marrow-derived vascular progenitor cells to areas of ischemia (Helisch and Schaper 2003, Voskuil, van Royen et al. 2003, Urbich and Dimmeler 2004, Park, Hoffman et al. 2010). Nitric oxide (NO) is a critical mediator of each of these mechanisms. Stenosis or occlusions of lower limb arteries promote changes in shear stress in pre-existing small arteries and arterioles. Increased shear stress within collateral arteries leads to NO induced vasodilation that results in an increase in flow through the collaterals. Elevated shear stress promotes inflammation, recruitment of monocytes and release of various growth factors such as platelet-derived growth factors (PDGFs) and monocyte chemoattractant protein (MCP)-1, which promote remodelling of arteries (Helisch and Schaper 2003, Voskuil, van Royen et al. 2003). The remodelled arteries have enhanced pericyte coverage and increased diameter. Furthermore, vascular progenitor cells participate in collateral artery remodelling, wherein net conductance across the collateral vessels is increased compensating to some extent for the occluded arteries (Helisch and Schaper 2003, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016).

In PAD, neo-vascularisation in ischaemic tissues located distal to the occlusion commonly occurs through a process referred to as angiogenesis (Niiyama, Huang et al. 2009). Angiogenesis is the expansion of the microvasculature because of sprouting of endothelial cells from pre-existing capillaries. The sprouting of a new capillary network is mediated through the activation, proliferation and migration of endothelial cells, extracellular proteolysis and vascular wall remodelling. A number of growth factors, such as hypoxia-inducible growth factors (HIF-1) and vascular endothelial growth factor (VEGF), as well as cytokines play a

major role in this process. This eventually results in the formation of new capillaries (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016).

In PAD, the efficiency of arteriogenesis and angiogenesis to restore blood supply is dramatically decreased in the presence of co-morbidities such as old age and risk factors (Dragneva, Korpisalo et al. 2013, Heuslein, Murrell et al. 2016, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Collateral blood flow after major artery occlusion promoted by arteriogenesis and angiogenesis may be sufficient in some patients to meet ischaemic skeletal muscle needs at rest. However, collateral circulation is generally not sufficient to meet oxygen consumption during exercise, which profoundly limits patient's physiological activity and quality of life (Sanada, Kanbara et al. 2016).

In addition, to atherosclerotic occlusions, blood supply to ischaemic tissues is further hampered by endothelial and microcirculatory dysfunction. Episodes of ischaemia-reperfusion caused by atherosclerotic occlusions leads to inflammation, and oxidant stress within the lower limb muscles (Schellong, Boger et al. 1997, Bragadeesh, Sari et al. 2005, Brevetti, Giugliano et al. 2010, Brass 2013, Hiatt, Armstrong et al. 2015). Inflammatory mediators can aggravate endothelial dysfunction and markers, such as interleukin-6, are inversely correlated with maximum treadmill performance (Nylaende, Kroese et al. 2006, Hiatt, Armstrong et al. 2015). During ischemia, skeletal muscle mitochondria release free radicals, including superoxide and other reactive oxygen species (ROS), that are derived from the oxidation–reduction cascade (Nylaende, Kroese et al. 2006). Reperfusion of ischaemic muscle after exercise leads to increase in oxidant stress (Hickman, Harrison et al. 1994). These ROS trigger numerous pathophysiologic pathways, including endothelial dysfunction (Nylaende, Kroese et al. 2006, Hiatt, Armstrong et al. 2015).

Impaired endothelial function and impaired microvascular perfusion as assessed by flow-mediated dilation and cuff occlusion has been correlated with the clinical severity of PAD (Coutinho, Rooke et al. 2011, Grenon, Chong et al. 2014, Meneses, Nam et al. 2018). Endothelial dysfunction is associated with walking impairment independent of the severity of impaired limb blood flow (as assessed by ABPI), suggesting that endothelial dysfunction may contribute to the exercise impairment in PAD (Hamburg and Balady 2011, Grenon, Chong et al. 2014). In a large cohort of patients from the Edinburgh Artery Study, blood viscosity and plasma fibrinogen were independently associated with the hemodynamic severity of PAD,

suggesting that increased blood viscosity also contributes to the symptoms of PAD (Fowkes, Housley et al. 1991, Coutinho, Rooke et al. 2011, Hiatt, Armstrong et al. 2015).

Evidence suggests that in addition to impaired vasculature, skeletal muscle pathophysiologic changes contribute to the lower extremity functional impairment and functional decline that is present in PAD (Hiatt, Armstrong et al. 2015, McDermott 2015, Treat-Jacobson, McDermott et al. 2019). Numerous pathological changes have been identified in the skeletal muscle of patients with PAD, including muscle apoptosis and atrophy, increased fibre type switching, altered myosin heavy-chain expression, and muscle fibre denervation (McGuigan, Bronks et al. 2001, Askew, Green et al. 2005, McDermott, Hoff et al. 2007, Mitchell, Duscha et al. 2007). These structural changes may be mediated, partly, by higher levels of inflammatory mediators in PAD (McDermott, Ferrucci et al. 2007). In the WALCS II cohort, including 380 PAD participants who were followed prospectively after baseline measurement of calf muscle characteristics, PAD participants with greater CT-measured calf muscle percent fat and lower CT-measured calf muscle density at baseline each had an increased incidence of mobility loss at 2-year follow-up (McDermott, Ferrucci et al. 2009). PAD participants in the lowest tertile of calf muscle density had a 3.50-fold increased hazard of mobility loss at two-year follow-up, compared to PAD participants in the highest tertile of calf muscle density at baseline. These associations were independent of age, sex, race, comorbidities, smoking, BMI, and ABPI (McDermott, Ferrucci et al. 2009). This demonstrates that ischemia related pathophysiologic changes in lower extremity calf skeletal muscle predicts increased rates of mobility loss (McDermott 2015).

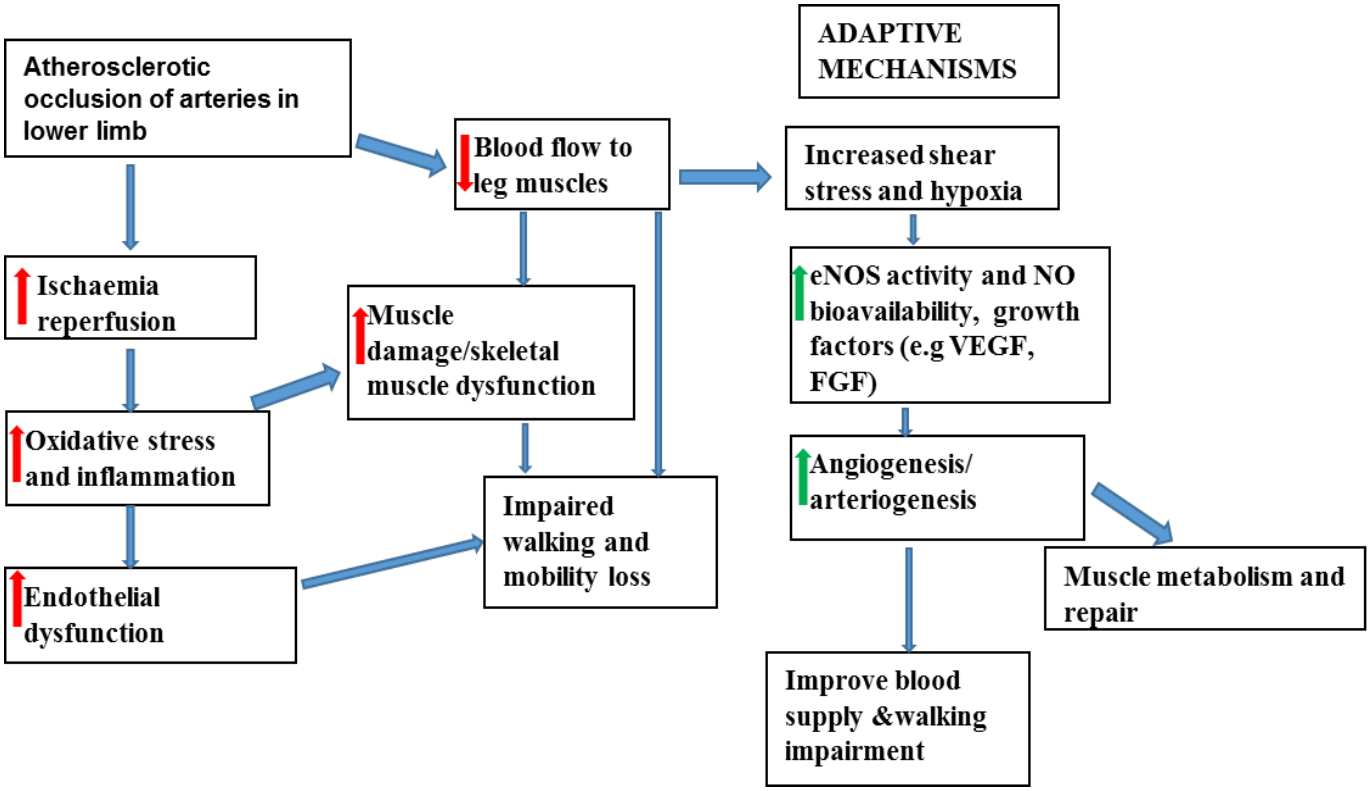


Figure I1. Pathophysiology of walking impairment in PAD. Figure shows adaptive mechanisms inadequate to compensate for the limited blood supply.

1.4.2 Treatments for limb ischaemia in PAD patients

1.4.2a Goal of treatment and outcome measures

PAD affects functional performance, independence in daily living and thus quality of life (Gerhard-Herman, Gornik et al. 2016). The quality of life of PAD patients centres on their walking impairment and patients with CLI are concerned with rest pain and the risk of limb amputation (Kinlay 2013, McDermott 2018). For patients with IC, improving walking performance and preventing progression to adverse limb related events is a major goal of treatment (McDermott 2018). In CLI patients, resolution of rest pain, complete ulcer healing, and avoidance of amputation are major treatment aims (Kinlay 2013).

Treatment efficacy in PAD patients is objectively measured by functional tests (treadmill walking test and six-minute walking tests) and haemodynamic tests. In the treatment of CLI, guidelines support primary end points of complete resolution of rest pain, complete ulcer healing, and avoidance of major amputation (Labs, Dormandy et al. 1999, Kinlay 2013).

The American Heart Association/American College of Cardiology clinical practice guidelines for PAD recommend both treadmill exercise testing (Class I recommendation, Level of Evidence-B) and six-minute walk testing (Class IIb recommendation, Level of Evidence-B) for objective assessment of changes in walking performance in patients with PAD (Hirsch, Haskal et al. 2006, McDermott 2015). Treadmill exercise testing is the most commonly used objective outcome measure in randomised clinical trials studying interventions to improve walking performance in people with PAD (Treat-Jacobson, McDermott et al. 2019).

Constant load treadmill protocols where the patient is required to walk as far as possible at a set speed and grade are most commonly used in the clinical setting and enable the response to an absolute workload to be established and then reassessed after treatment (Kinlay 2013, Askew, Parmenter et al. 2014). The six-minute walk test has been well validated in patients with PAD (McDermott, Liu et al. 2013, McDermott, Guralnik et al. 2014, McDermott 2015). The six-minute walk is usually performed on a 100-foot corridor in which the distance covered by walking back and forth over 6 minutes is measured (Kinlay 2013, McDermott 2015).

1.4.2b Surgery

Indications for surgical revascularisation in patients with PAD are ischaemic rest pain, ischaemic ulcers or gangrene, and severe functional disability that interferes with the patient's lifestyle (Gerhard-Herman, Gornik et al. 2016, Aboyans, Ricco et al. 2018, Golledge, Moxon et al. 2018). Endovascular or surgical revascularisation of the limb to improve limb circulation and for limb salvage plays a key role in the management of PAD (Conte and Pomposelli 2015, Gerhard-Herman, Gornik et al. 2016, Meneses, Ritti-Dias et al. 2017). Surgical approaches to restoring the arterial blood supply to the legs involves using temporary balloons, permanent stents or open surgical bypasses (Russell, Homer-Vanniasinkam et al. 2012). Stents and other peripheral revascularisation procedures have a risk of serious complications (such as major amputation and death) and poor long-term durability (Golledge, Moxon et al. 2018). Hence, revascularisation interventions that ensure stable, viable and durable vasculature for patients with advanced PAD are sought (Bonaca and Creager 2015, Gerhard-Herman, Gornik et al. 2016).

1.4.2c Exercise treatment

Exercise therapy is currently recommended as part of the initial treatment for all patients with IC. Exercise is the most effective non-invasive therapy to improve pain symptoms and ambulation in IC. A considerable body of evidence and clinical guidelines support the

management of patients by supervised treadmill exercise and home-based exercise that incorporate behavioural change techniques significantly improve pain-free and maximal walking distance in people with PAD (McDermott, Ades et al. 2009, Gardner, Parker et al. 2011, Hamburg and Balady 2011, Fakhry, van de Luijtgaarden et al. 2012, McDermott, Liu et al. 2013, Askew, Parmenter et al. 2014, Gardner, Parker et al. 2014, McDermott, Ferrucci et al. 2017, McDermott 2018, Golledge, Singh et al. 2019). The European Society of Cardiology, American Heart Association and Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease have all declared that the evidence supporting supervised exercise therapy in the treatment of IC is sufficiently robust to merit a Level I recommendation (Norgren, Hiatt et al. 2007, European Stroke, Tendera et al. 2011, Brass 2013, Gerhard-Herman, Gornik et al. 2016).

Supervised exercise programmes usually consist of three or four supervised exercise sessions per week at a central facility (Golledge, Singh et al. 2019). These programmes are, however, frequently not taken up when offered to patients with PAD, in part owing to the impracticality of attending a central facility (Makris, Lattimer et al. 2012, Askew, Parmenter et al. 2014, Harwood, Smith et al. 2016, Golledge, Singh et al. 2019). Lack of reimbursement is an additional barrier to utilisation of this treatment (Berger Jeffrey and Hiatt William 2012). Furthermore, supervised exercise programmes are not available in most parts of the world possibly because of limited evidence on long-term effects (Regensteiner 2004, Morris, Rodriguez et al. 2014)

There is increasing attention to alternative structured exercise programs that avoid frequent traveling to a medical centre. Structured community or home-based exercise programs using clinician-supported and instruction-based techniques have been shown to be effective at improving walking performance (McDermott, Spring et al. 2018, Pymer, Tew et al. 2018, Golledge, Singh et al. 2019). Other walking exercise programs such as Nordic walking uses poles and a core-focused walking technique to reduce the load on the legs. Walking improvements have been suggested to be similar for patients treated by Nordic and standard walking programmes (Oakley, Spafford et al. 2017, Golledge, Maarij et al. 2018, Golledge, Moxon et al. 2018). Other non-walking based, modalities have also been studied, including cycling, strength training, and arm cranking. These could be useful when walking exercise is not considered viable. More evidence is needed to clarify differences in training methods (Parvar, Fitridge et al. 2018).

The mechanisms that lead to improvement in walking ability after exercise training are poorly understood (Hamburg and Balady 2011, McDermott 2018, Treat-Jacobson, McDermott et al. 2019). A combination of adaptive mechanisms account for the benefits after training. These act by reversing some of the pathophysiological processes underlying the condition (Hamburg and Balady 2011, Treat-Jacobson, McDermott et al. 2019). The majority of studies in patients that have IC have shown little or no increase in blood flow assessed by ABPI following an exercise programme, even when significant improvements in walking ability have been reported (Stewart , Hiatt et al. 2002, Duscha, Robbins et al. 2011, Meneses, Nam et al. 2018). Studies have consistently shown a poor correlation between leg blood flow assessed by ABPI and walking ability (Parmenter, Raymond et al. 2010, Duscha, Robbins et al. 2011, Askew, Parmenter et al. 2014, Meneses, Ritti-Dias et al. 2017, Meneses, Nam et al. 2018). As there is currently little evidence to support an increase in blood flow as a major factor in the increase in walking ability after training, other mechanisms may account for the improvements seen such as improvements in endothelial function and skeletal muscle metabolism and function (Menard, Smith et al. 2004, Hiatt, Armstrong et al. 2015, McDermott 2018).

1.4.2d Pharmacotherapy for limb ischaemia

Cilostazol is the only Federal Drug Administration approved medication for PAD related ischaemic symptoms that is recommended by clinical practice guidelines (Sanada, Kanbara et al. 2016, McDermott 2018). Cilostozol is a phosphodiesterase inhibitor, which improves blood supply to ischaemic muscles and provides approximately 25 to 40% improvement in treadmill walking performance in patients with PAD (Gresele, Momi et al. 2011). Despite this, cilostozol is not publicly funded in Australia as the benefit was deemed to be too modest (Bedenis, Stewart et al. 2014, Golledge, Moxon et al. 2018). More effective medications are sought to improve blood supply and limb symptoms.

The mechanisms by which cilostozol causes improvement in walking ability in patients with PAD is unclear. Antiplatelet and vasodilatory effects have been suggested to be the most likely causes of cilostozol's mechanism of action. Cilostazol inhibits both cyclic nucleotide phosphodiesterase type 3 (PDE3) and adenosine uptake (Liu, Shakur et al. 2001). PDE3 inhibition elevates intracellular cyclic adenosine monophosphate (cAMP) and the inhibition of adenosine uptake elevates interstitial and circulatory adenosine concentration (Sanada, Kanbara et al. 2016). In platelets and vascular smooth muscle cells, adenosine increases intracellular cAMP by activating adenosine A2 receptors. Simultaneous inhibition of PDE3

and elevation of extracellular adenosine causes a synergistic increase in the concentration of cAMP within platelets and vascular smooth muscle cells, resulting in potent antiplatelet and vasodilatory effect (Liu, Shakur et al. 2001). Despite the benefits of cilostazol in improving quality of life in patients, it is contraindicated in patients with severe renal impairment, moderate or severe hepatic impairment, and known predisposition to bleeding and in patients with history of ventricular tachycardia, ventricular fibrillation or multifocal ventricular ectopic beats, or prolongation of the QTc interval (Real, Serna et al. 2018). Cilostazol has been associated with a number of spontaneous reports of MI, angina, arrhythmias and serious bleeding (EMA 2013, Real, Serna et al. 2018). Haemorrhagic events in elderly patients co-treated with antiplatelets have also been reported (EMA 2013). Alternative effective pharmacological options are sought for PAD patients due to the concerns of safety with cilostozol.

Interestingly, in pre-clinical studies, cilostozol has been shown to promote neo-vascularisation in response to HLI via an e-NOS-dependent mechanism (Hori, Shibata et al. 2012). It has been suggested cilostozol promotes NO production by e-NOS activation via cAMP/PKA- and PI3K/Akt-dependent mechanisms (Hashimoto, Miyakoda et al. 2006, Biscetti, Ferraccioli et al. 2015).

1.4.2e A potential target for future drug therapy in limb ischaemia

Current PAD therapies are associated with significant limitations. Cilostozol may not achieve an ideal response rate, and supervised exercise efficacy may be limited by co-morbidities and lack of access to facilities (Yoshida, Horimoto et al. 2003). Furthermore, primary endovascular or open surgical revascularisation may not be feasible, durable or cost-effective. Urgent medical options are an unmet need for limb ischaemia in PAD patients and targets for effective medical therapy are required. NO is a potential target for revascularisation in limb ischaemia.

Patients with PAD lack the ability to endogenously increase vascular NO bioavailability, leading to significant dysfunctions within the vasculature and ischaemic muscles (Woessner, VanBruggen et al. 2017). NO is a critical vasoactive substance which regulates the tissue response to ischaemia by mediating arteriogenesis and angiogenesis. Three isoforms of nitric oxide synthases (NOS) generate NO. NO is derived primarily from the activity of endothelial NOS (e-NOS). The two other isoforms of NOS from which NO is derived are neuronal NOS and inducible NOS. The oxidoreductase e-NOS catalyses the formation of NO from L-arginine and oxygen in a reaction requiring calcium/calmodulin, flavin mononucleotide, flavin adenine

dinucleotide, nicotinamide adenine dinucleotide phosphate (NADPH) and regulatory co-factors such as tetrahydrobiopterin (BH4) or heat shock protein-90 (HSP90) (Griffith and Stuehr 1995, Xia, Tsai et al. 1998, Lin, Lin et al. 2004, Sessa 2004).

Dimerisation promoted by regulatory proteins is required for e-NOS to produce NO. Monomerisation alters function of e-NOS resulting in the production of superoxide instead of NO. This alteration is referred to as "e-NOS uncoupling" and is a major cause of endothelial dysfunction (Vasquez-Vivar, Kalyanaraman et al. 1998, Chen, Druhan et al. 2008).

Phosphorylation of e-NOS at Ser 1177 enhances e-NOS enzyme activity to increase NO bioavailability (Dimmeler, Fleming et al. 1999, Fulton, Gratton et al. 1999). Phosphorylation of serine 1177 is catalysed by a number of distinct kinases including Akt, protein kinase A (Chen, Mitchelhill et al. 1999, Bauer, Fulton et al. 2003), 5' adenosine monophosphate-activated protein kinase, calcium-calmodulin kinase and checkpoint kinase-l (Butt, Bernhardt et al. 2000, Boo, Sorescu et al. 2002, Chen, Druhan et al. 2008, Heiss and Dirsch 2014). NO signalling is classically characterised to occur by its interaction with the iron heme in soluble guanylate cyclase, causing its activation as the enzyme to produce guanosine 3'-5'-monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). cGMP activates several protein kinases subsequently leading to arteriogenesis and angiogenesis (Allen, Giordano et al. 2012). Treatments that modulate e-NOS activity to increase NO bioavailability could have major therapeutic benefits for patients with PAD (Falconer, Papageorgiou et al. 2018). Furthermore, revascularisation after induction of acute HLI is severely impaired in e-NOS deficient mice resulting in poor blood flow recovery and severe limb ischemia (Yu, deMuinck et al. 2005, Kondo, Shibata et al. 2009, Takahashi, Shibata et al. 2015) suggesting that e-NOS plays a key role in promoting revascularisation in limb ischaemia. Therefore, drugs which modulate e-NOS activity and endogenous modulators of e-NOS have potential to play key therapeutic roles in limb ischaemia.

1.5 Novel treatments tested for PAD

Numerous pre-clinical studies have shown promising efficacy of a several therapeutic agents in restoring blood supply to limb ischaemia (Table 1). However, translation of these studies have been disappointing in clinical trials (Table 1). Clinical trials have not shown improvements in primary outcome measures such as improved exercise performance, decreased rates of amputation, or decreased mortality (Lederman, Mendelsohn et al. 2002, Rajagopalan, Mohler et al. 2003, Kusumanto, van Weel et al. 2006, Powell, Simons et al. 2008, Tongers, Roncalli et al. 2008, Belch, Hiatt et al. 2011, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016).

Pre-clinical studies have suggested that novel cellular and molecular therapies stimulate the development of new blood vessels in the models tested (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). Several interventions, such as of bone marrow mononuclear cells (BM-MNC), bone marrow-derived aldehyde dehydrogenase bright cells (ALDH br), granulocyte-macrophage colony-stimulating factor (GM-CSF), gene transfer of fibroblast growth factor (FGF) using plasmid based delivery system (NV1FGF), VEGF, HGF and development-regulated endothelial locus-1 (Del-1) have been successful in animal models (Takeshita, Zheng et al. 1994, Tabata, Silver et al. 1997, Morishita, Nakamura et al. 1999, Ojalvo, Seralena et al. 2003, Yoshida, Horimoto et al. 2003, Zhong, Eliceiri et al. 2003, Fujii, Yonemitsu et al. 2006, Capoccia, Robson et al. 2009, Yonemitsu, Matsumoto et al. 2013, Kuwahara, Nishinakamura et al. 2014).

In the phase II placebo-compared randomised-controlled trial PACE (Patients with Intermittent Claudication Injected with ALDH Bright Cells), patients with claudication and infra-inguinal PAD received autologous ALDHbr cells (n=38) or placebo (n=40). All patients received ten injections of placebo or ALDHBr cells into the thigh and calf of the index leg. Follow up in the PACE trial after six months, showed no improvement in peak walking time, collateral count, peak hyperaemic popliteal flow, and capillary blood supply in patients treated by autologous bone marrow-derived ALDHBr cells (Perin, Murphy et al. 2017).

The phase II randomised-controlled trial PROVASA (Intra-arterial Progenitor Cell Transplantation of Bone Marrow Mononuclear Cells for Induction of Neovascularisation in Patients with Peripheral Arterial Occlusive Disease), 40 patients with CLI received either intra-arterial BM-MNC (n=19) or placebo (n=21), and after 3 months, both groups were treated with BM-MNC. There was no difference in ABPI or limb salvage at 3 or 6 months (Walter,

Krankenberg et al. 2011). In the phase II randomised-controlled trial JUVENTAS (Rejuvenation of endothelial progenitor cells via transcutaneous intra-arterial supplementation), 160 patients with CLI received 3 separate intra-arterial infusions of BM-MNC (n=81) versus placebo (n=79), at 3-week intervals, into the common FA of the ischaemic limb. There were no significant differences in the primary endpoint of 6-month major amputation rate or the secondary endpoints of quality of life, rest pain, ABPI, or TcPO2 (Teraa, Sprengers et al. 2015).

The PROPEL (Progenitor Cell Release Plus Exercise to Improve Functional Performance in PAD) randomised trial examined whether GM-CSF combined with supervised treadmill walking exercise improves six-minute walk distance more than GM-CSF alone, more than supervised treadmill exercise alone, and more than placebo in participants with PAD. Combining GM-CSF with supervised treadmill exercise did not significantly improve six-minute walk distance more than supervised exercise alone or more than GM-CSF alone. GM-CSF alone did not significantly improve six-minute walk distance more than placebo (McDermott, Ferrucci et al. 2017).

Intra-arterial administration of recombinant fibroblast growth factor (FGF) has been reported to be beneficial in patients with PAD in the Therapeutic Angiogenesis with FGF-2 for intermittent claudication (TRAFFIC) study (Lederman, Mendelsohn et al. 2002). However, the phase III Therapeutic Angiogenesis for the Management of Arteriopathy in a Randomised International Study (TAMARIS) involving patients from 30 different countries, suggested that non-viral-FGF gene therapy was not effective in reducing major amputation or death in PAD patients (Belch, Hiatt et al. 2011).
The double-blinded randomised placebo-controlled phase II study named Regional Angiogenesis with VEGF in PAD (RAVE) showed no improvement in the primary efficacy end-point of walking time (Rajagopalan, Mohler et al. 2003).

Trials with plasmid-encoding human HGF in CLI patients have demonstrated no difference in ABPI, toe-brachial index, pain relief, wound healing, or major amputation (Powell, Simons et al. 2008, Shigematsu, Yasuda et al. 2010).

A double-blinded placebo-controlled study assessing the effect of plasmid-encoded angiomatrix protein Del-1 for the treatment of IC [Del-1 for Therapeutic Angiogenesis Trial (DELTA-1)] also did not find any benefit in the main outcomes assessed such as peak walking time (Grossman, Mendelsohn et al. 2007).

Several explanations have been provided to account for the lack of efficacy in clinical trials. These include inadequate therapeutic doses, insufficient duration of exposure, compromised delivery and poor vector transduction efficiency (Gupta, Tongers et al. 2009, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). Methodological flaws in clinical studies include small sample size, lack of reproducible assessments and short duration of testing. In addition, potential patient-related issues proposed to underlie inefficacy include defects in the response to angiogenic stimuli due to existing comorbidities, the use of other medications, circulating angiogenic inhibitors, lack of target receptor expression in target tissues, lack of viable muscle tissue required for a therapeutic response, and growth factor resistance in a chronically ischemic environment (Dragneva, Korpisalo et al. 2013).

A major area of concern which accounts for the lack of efficacy in clinical trials is the overoptimistic conclusions derived from interventions examined in pre-clinical studies (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Animal models that appropriately recapitulate key features of the human disease and improved pre-clinical study outcome measures and study designs could be essential prerequisites to progress in developing treatments for PAD (Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016)

Table I1: Examples of cellular and molecular interventions tested in unilateral HLI animal models and clinical trials						
Cell-type tested	**Pre-clinical Unilateral HLI model used**	**Animal used for HLI induction**	**Sample sizes/Groups**	**Main outcome assessed in animal models**	**Results of animal studies/end-points assessed**	**Clinical Outcomes of clinical trials**
BM-MNC	Excision of the FA (Yoshida, Horimoto et al. 2003)	Lewis rats	BM-MNCs ($5x10^6$ cells; n=6) or PBS (n=7) BM-MNCs ($3x10^7$ cells; n 6) or PBS (n=7)	Angiography/ IHC	Significant development of collateral vessels in both BM-MNC-transplanted groups compared to controls. Capillary endothelial cells were increased in both BM-MNC-transplanted groups.	Phase II randomised-controlled PROVOSA trial: CLI patients treated with either intra-arterial BM-MNC (n=19) or placebo (n=21) 3 months, and after 3 months, both groups were treated with BM-MNC. There was no difference in ABPI or limb salvage at 3 or 6 months difference in ABPI, amputation or death (Walter, Krankenberg et al. 2011). Phase II randomised-controlled JUVENTAS trial in CLI patients treated with BM-MNC (n=81) versus placebo (n=79): showed no difference in amputation, death, ABPI, ulcer size, quality of life, rest pain, TcPO2 (Teraa, Sprengers et al. 2015).
BM-derived aldehyde dehydrogenase bright cells	Ligation and excision of FA (Capoccia,	NOD/SCID β2M null or NOD/SCID/MPSVII mice	Tail vein injection of PBS (n = 8), 50×10^6 BM MNCs	LDI and IHC	BM-MNC administration improved blood	Phase II placebo-compared randomised-controlled trial PACE

	Robson et al. 2009)		(n = 6), 5×10^5 $ALDH^{lo}$ (n = 8), or 1 to 2×10^5 $ALDH^{hi}$ cells (n = 8)		supply and capillary density in mice treated with ALDH Br cells	on IC patients treated with ALDHbr cells (n=38) compared to placebo controls (n=40) did not improve PWT or magnetic resonance outcomes, and the changes in PWT were not associated with the anatomic or physiological magnetic resonance imaging end points. (Perin, Murphy et al. 2017)
GM-CSF	Excision of the FA and vein (Kuwahara, Nishinakamura et al. 2014)	C57BL/6N mice	GM-CSF (n=5–7) and PBS (n=5–7)	LDI	Blood flow recovery was significantly improved in mice treated with GM-CSF-dependent BMCs (Kuwahara, Nishinakamura et al. 2014)	GM-CSF did not significantly improve six-minute walk distance more than placebo. Combining GM-CSF with supervised treadmill exercise did not significantly improve six-minute walk distance more than supervised exercise alone or more than GM-CSF alone. (McDermott, Ferrucci et al. 2017)
HGF	Ligation and excision of FA (Morishita, Nakamura et al. 1999, Taniyama,	NZW rabbits	Control (n=7–8), 100 μg of hHGF vector transfection (n=7–8), 250 μg of hHGF vector transfection (n =	Quantitative angiography, intra-arterial guided wire measurement of	Increased blood supply, CBP ratio and collateral vessel development from the origin stem artery to the distal	Phase I/II study of HGF plasmid-treated CLI patients (n = 78) compared with placebo-treated CLI patients (n = 26)

	Morishita et al. 2001)		7–8), 500 µg of hHGF vector transfected HGF group (n=7–8)	blood flow and CBP ratio	point of the reconstituted parent vessel was observed in rabbits receiving IM injection of hHGF plasmid once, 10 days after surgery	showed no differences were observed in the toe-brachial index, ABPI, pain relief, wound healing or major amputation (Powell, Simons et al. 2008) Phase II/III of HGF plasmid-treated CLI patients (n=27) with placebo treated CLI patients (n=13) showed no difference in rest pain, ABPI or amputation (Shigematsu, Yasuda et al. 2010)
		Sprague–Dawley rats	Control (n=5–8), 100 µg of hHGF vector transfection (n=5–8), 250 µg of hHGF vector transfection (n = 5–8) and 500 µg of hHGF vector transfected (n= 5–8)	LDI and capillary density by IHC with alkaline phosphatase staining of ECs	A dose-dependent increase in blood supply was observed in both HGF and hHGF transfection	
FGF (1 and2)	Ligation and excision of FA and femoral vein (Masaki, Yonemitsu et al. 2002)	C57BL/6 and BALB/c nu/nu mice	PBS control (n=10), SeV luciferase control (n=10), SeV–FGF2 (n =10) and SeV–VEGF^{165} (n= 10)	LDI, limb salvage score and IHC	Increased blood supply, increased capillary densities and limb salvage with IM injection of SeV–FGF-2 compared with control vector, PBS and SeV–VEGF^{165} accelerated amputation, massive muscular oedema, necrosis and disturbed regeneration with IM injection of VEGF^{165}; increased number of capillaries with	Phase I/IIa clinical trial showed that DVC1-0101, a new vector-based human FGF-2 gene transfer (rSeV/dF–hFGF2), in PAD patients (n = 12) with rest pain was safe and well tolerated and resulted in improvement of limb function (Yonemitsu, Matsumoto et al. 2013) Phase II trial (TRAFFIC) in recombinant FGF-2 treated PAD patients (n=127) resulted in improved PWT and

					no improvement in blood supply	ABPI compared with placebo-treated group (n=63) (Lederman, Mendelsohn et al. 2002) Phase III TAMARIS trial in CLI patients treated with non-viral-FGF gene (n=259) compared to placebo (n=256) showed no difference in amputation or death
	Ligation and excision of FA and femoral vein and their branches up to and including the SA and PA bifurcation (Fujii, Yonemitsu et al. 2006)	CCR deficient and BALB/c nu/nu mice	Luciferase control (n=10), SeV luciferase (n = 30), SeV mFGF-2(30), mFGF-2 luciferase (n=10), mFGF-2 7ND MCP-1 (n=10) and luciferase7ND MCP-1 (n=10)	LDI and IHC	Dominant negative mutant-MCP-1 gene transfer diminished both adaptive and FGF-2-mediated recovery of blood flow; CCR2-deficient mice lost approximately40 % of their limbs compared with controls; SeV-mediated FGF2 gene transfer significantly but partially restored the limb survival of CCR2-deficient mice	
	Ligation and excision of FA (Tabata, Silver et al. 1997)	NZW rabbits	pGSVLacZ control (n=10) non-secreted aFGF (p267, n=10), secreted aFGF (pMJ35, n=10)	CBP ratio, internal iliac arteriography, capillary density and regional	pMJ35 transfectants had increased angiographically visible collaterals, CBP ratio and	

				blood flow using coloured microspheres	capillary density and lower vascular resistance than either p267 or LacZ control	
VEGF	Ligation and excision of FA (Pu, Sniderman et al. 1993, Takeshita, Zheng et al. 1994)	NZW rabbits	Saline control (n =11) and EC growth factor (n=11)	CBP ratio, 99mTC macro-aggregate calf radioisotopic perfusion scan, angiography	Increased blood supply, CBP ratio and increased revascularisation in growth factor-treated animals	Phase II clinical trial (RAVE) showed that treatment with $AdVEGF^{121}$ in PAD patients (n=72) did not improve PWT, quality of life and claudication onset time (Rajagopalan, Mohler et al. 2003)
	Ligation of CIA (Makinen, Manninen et al. 2002)	Sprague–Dawley rats	PBS control (n=8), naive control (n=8), AdVEGF treated (n=8) and AdNull (n =8)	Blood flow imaging by Colour microsphere and 99mTC-labelled sestamib radionucleotide, angiography and Histology	Increased blood supply and greater vascularity in the Ad-VEGF-treated group	
	Ligation and excision of FA (Mack, Magovern et al. 1998, Gowdak, Poliakova et al. 2000)	Wistar rats	PBS control (n=3), AdCMV–$VEGF^{121}$(n=6) and AdCMV.null (n=5)	Bioenergetic reserve by NMR spectroscopy and IHC	AdCMV–$VEGF^{121}$ -treated animals showed markedly improved bioenergetic reserve, capillary density, tissue blood supply and spontaneous collateral vessel development	
	Ligation and excision of FA (Ojalvo, Seralena et al. 2003)	Beagle dogs	Saline control (n=6) and $VEGF^{121}$ plasmid treated (n= 6)	CBP ratio, angiography, vasomotor reserve and haematological assays	Increased collateral artery development, CBP ratio and blood supply in $pVEGF^{121}$ -treated groups. No significant	

					modification in haematological variables	
Del-1	Ligation and excision of FA (Zhong, Eliceiri et al. 2003)	NZW rabbits	Non-coding plasmids control (n=3), $hVEGF^{165}$ plasmid (n = 6) and hDel-1 plasmid (n=4)	Angiography, gene expression by RT-PCR and CD31 expression by IHC	hVEGF and hDel-1 plasmid induced the formation of 3-fold more new blood vessels	Phase II clinical trial showed no improvements in PWT, claudication onset time, ABPI in Del-1 plasmid-treated IC patients (n=52) and placebo-treated IC patients (n=53) There were no significant differences between groups (Grossman, Mendelsohn et al. 2007)
	Ligation and excision of FA (Zhong, Eliceiri et al. 2003)	NZW rabbits	Non-coding plasmids control (n=3), $hVEGF^{165}$ plasmid (n=6) and hDel-1 plasmid (n=4)	Angiography, gene expression by RT-PCR and CD31 expression by IHC	hVEGF and hDel-1 plasmid induced the formation of 3-fold more new blood vessels	
	Ligation of FA (Zhong, Eliceiri et al. 2003)	CD1 mice	Non-coding plasmid control (n=7–8), $hVEGF^{165}$plasmid(n = 7–8) and hDel-1 plasmid (n=7–8)	Treadmill run time, capillary myofibre ratio determination by IHC with CD31, Del-1 and $VEGF^{165}$	hVEGF and Del-1 were equally effective in inducing neovessel formation and restoring hind-limb function	

Abbreviations: BM-MNC, bone marrow-derived mononuclear cells; CBP, calf blood pressure ratio; CCR, CC chemokine receptor; CD, cluster of differentiation; CIA, common iliac artery; FA, femoral artery, DFA, deep FA; hADSC, human adipose-derived stem cells; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; IHC, immunohistochemistry; IM, intramuscular; NZW, New Zealand white; PWT, peak walking time; SVF, stromal vascular fraction; VEC; vascular EC; $TcPO_2$, transcutaneous blood pressure index; SeV, Sendai virus; 99mTC, technetium; AdCMV, adenoviral cytomegalovirus vector; Del-1, delta-1 .

1.6 Animal models of HLI

Pre-clinical animal models are indispensable research tools for the development of novel therapies, the assessment of medical interventions and the study of molecular pathways involved in disease development. A suitable animal model is potentially important to improve translation of pre-clinical findings. Large and small animals have been used as research tools to model PAD in pre-clinical experiments (Waters, Terjung et al. 2004). Large animal models benefit from the ease of accurate identification of the lower extremity inflow vessels and their branches, as well as a multitude of blood flow measures that can be used throughout the course of a study. Usually small animal models, mainly genetically modified mice strains, are preferred due to the availability of transgenic strains enabling assessment of the effects of genetic deficiency or overexpression (Hoefer, van Royen et al. 2004). The availability of large numbers of knockout and transgenic strains, a well characterised genome, ease of maintenance and handling and the ability to multiply the breeding stock in limited time, makes it less challenging to plan and conduct experiments in mice (Field 1993, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016).

Several mouse models have been developed to mimic limb ischaemia of PAD patients. (Couffinhal, Silver et al. 1998, Nowak-Sliwinska, Alitalo et al. 2018). Several different aspects that have varied including mouse strain, method of ischaemic induction, risk factors and monitoring.

1.7 Common mouse models of HLI

Unilateral mouse HLI induction has been usually used as a model to study PAD. This model permits the use of the contra-lateral extremity as a control within the same mouse. To generate models of HLI, several surgical techniques are used, which include the ligation of iliac artery, femoral artery (FA), FA and vein ligation, or a combination with multiple ligation sites. The surgical procedures are performed under continuous infusion of anaesthetics such as isoflurane and the animals generally recover within minutes (Krishna, Omer et al. 2016). The degree of ischaemia obtained using the various surgical procedures depends on the type of surgery, occlusion site and the extent of lower limb artery involved. Typically, HLI is induced by various manipulations on the FA with or without the accompanying side branches as illustrated in Figure I1 (Pu, Sniderman et al. 1993, Couffinhal, Silver et al. 1998, Waters, Terjung et al. 2004, Madeddu, Emanueli et al. 2006, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Nowak-Sliwinska, Alitalo et al. 2018). It is reported that marked differences are observed

in the degree of ischaemia induced by each approach (Shireman and Quinones 2005, Goto, Fukuyama et al. 2006).

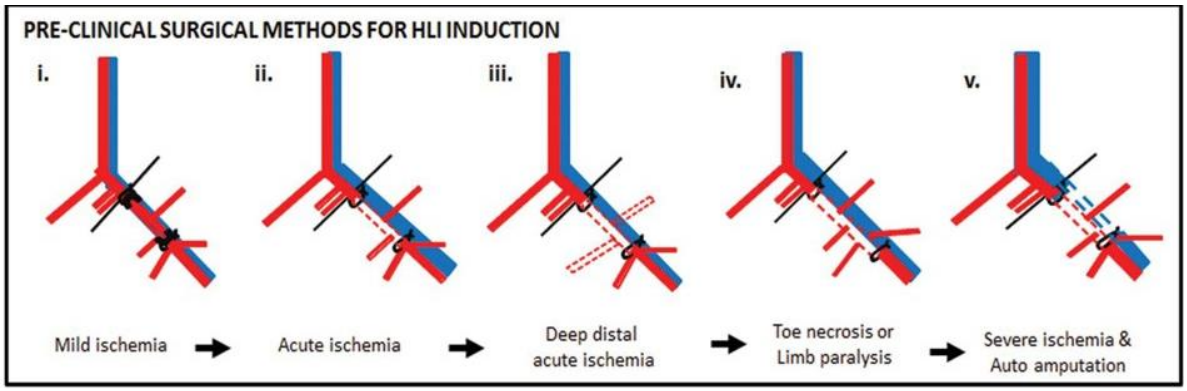


Figure I1. A simplified diagram showing various surgical techniques commonly employed involving the FA to induce unilateral HLI, the consequence and their relation to the presentation of lower extremity ischaemia. (i) Ligation of the FA proximally and distally using silk sutures, (ii) ligation of the FA proximally and distally plus excision of the intervening segment, (iii) ligation and excision of complete FA and its side branches, (iv) ligation of proximal FA and the distal saphenous artery and excision of the intervening segment, (iv) ligation and excision of both the FA and the femoral vein (Adapted from (Krishna, Omer et al. 2016)

1.7.1 Ligation model

The ligation of the FA results in milder ischaemia and blood flow is rapidly restored through collateral flow resulting with no or limited ischaemia or functional impairment observed (Figure I1Bi) (Couffinhal, Silver et al. 1999, Emanueli, Graiani et al. 2004, Goto, Fukuyama et al. 2006, Krishna, Omer et al. 2016).

1.7.2 Ligation and excision models

The ligation and excision of the complete FA and its side branches results in distal limb ischaemia with toe necrosis suggesting the length of occlusion is crucial for the degree of ischaemia (Figure I1Biii) (Couffinhal, Silver et al. 1998, Goto, Fukuyama et al. 2006, Krishna, Omer et al. 2016, Nowak-Sliwinska, Alitalo et al. 2018). Severe necrosis of the foot and/or limb paralysis is reported after ligation of the proximal FA and the distal saphenous artery in rodents (Madeddu, Emanueli et al. 2006) (Figure I1Biv). The excision of both the FA and femoral vein is reported to result in acute onset of severe ischaemia, necrosis of the toes, foot and knee and auto amputation. This approach does not reflect the pathophysiology and clinical presentations of CLI, since in patients, the symptoms are due to arterial obstruction and related

complications rather the venous involvement (Figure I1Bv) (Masaki, Yonemitsu et al. 2002, Goto, Fukuyama et al. 2006, Yu and Dardik 2018).

The most commonly reported method of ischaemia induction is ligation at the proximal and distal end of the FA followed by excision of the intervening segment (Figure I1Biii) (Couffinhal, Silver et al. 1998, Morishita, Nakamura et al. 1999, Madeddu, Emanueli et al. 2006, Krishna, Omer et al. 2016, Nowak-Sliwinska, Alitalo et al. 2018). This results in a moderate to severe limb ischaemia with variable muscle or toe necrosis (Brenes, Jadlowiec et al. 2012, Krishna, Omer et al. 2016). Additionally, acute ligation and excision usually results in natural rapid recovery of blood supply in the lower limb and does not reflect chronic ongoing limb ischaemia which is the most common presentation in patients (Chalothorn and Faber 2010, Nowak-Sliwinska, Alitalo et al. 2018).

1.7.3 Limitation of current models

The major drawback of currently used mouse models of HLI is the rapid recovery of blood supply in the ischaemic limb. Acute vessel occlusion in animal models result in recruitment of pre-existing collaterals to bypass the occlusion. The occlusion generates a pressure gradient between the proximal and distal ends of the occluded vessel, resulting in a redirection of blood flow towards the collaterals. Subsequently, the increased flow triggers a rise in collateral artery wall shear stress, alters endothelial gene expression and promotes rapid arteriogenesis (Pipp, Boehm et al. 2004, Heil, Eitenmuller et al. 2006, Dragneva, Korpisalo et al. 2013). Evidence in the literature illustrates the remarkably fast recovery of blood supply after sudden occlusion of a major artery in animal models of experimental ischemia, suggesting the existence of a strong endogenous compensatory collateral response (Tang, Chang et al. 2005, Yang, Tang et al. 2008, Dragneva, Korpisalo et al. 2013, Mohamed Omer, Krishna et al. 2016).

Furthermore, an acute arterial occlusion activates inflammatory responses within ischaemic tissues which may partially promote angiogenesis and muscle regeneration (Silvestre, Bergaya et al. 2001). The response involves: the recruitment and activation of inflammatory cells; VEGF-induced activation, proliferation and migration of endothelial cells; and activation of satellite cells within ischemia ischaemic muscles (Dragneva, Korpisalo et al. 2013). These events promote angiogenesis and regeneration of the ischaemic muscle. Thus, adaptive arteriogenesis and angiogenesis contribute to the recovery of blood flow and muscle function in animal models with acutely ischemia. In contrast, these endogenous recovery mechanisms

do not function sufficiently in patients with chronic ischaemic symptoms and co-morbidities (Dragneva, Korpisalo et al. 2013). The severity of experimental ischaemia induced also depends on the location and length of artery occlusion and also on the capacity for arteriogenesis and angiogenesis in the species used (Hellingman, Bastiaansen et al. 2010, Lotfi, Patel et al. 2013, Krishna, Omer et al. 2016, Thomas, Thirumaran et al. 2016, Nowak-Sliwinska, Alitalo et al. 2018).

1.7.4 Gradual constriction model of HLI

The major limitations of the currently used HLI models is the acute nature of generating ischemia and the rapid recovery of blood supply, whereas in the patients with PAD ischemia develops over several years and collaterals are inefficient in compensating ischaemic tissues (Aranguren, Verfaillie et al. 2009, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). The acute induction of HLI in current models result in sudden changes in fluid shear stress within collateral arteries to alter gene expression patterns through shear responsive elements. This subsequently promotes arteriogenesis and angiogenesis to restore blood supply to the ischaemic tissues (Topper and Gimbrone Jr 1999, Garcia-Cardena, Comander et al. 2001, Tang, Chang et al. 2005).

Ameroid constrictors have been used to model gradual stenosis of the FA. This approach has been used in rabbits, rats and mice (Baffour, Garb et al. 2000, Tang, Chang et al. 2005, Yang, Tang et al. 2008). An ameroid constrictor is an inner ring of casein that is surrounded by a stainless steel sheath. Casein is a hygroscopic substance that swells as it slowly absorbs body fluid. The stainless steel sheath forces the casein to swell inwardly. When placed on the FA *in vivo*, the inner diameter of the ameroid constrictor narrows gradually constricting the artery (Baffour, Garb et al. 2000, Tang, Chang et al. 2005, Yang, Tang et al. 2008). The nadir in blood supply within this model is gradually reached in 14 days after placing ameroids (Yang, Tang et al. 2008). The rate of blood flow recovery is poor with ameroids compared to the HLI model of FA ligation and excision (Yang, Tang et al. 2008). The expression levels of several genes crucial for the response to ischaemia (including HIF-1α, and VEGF) within the gastrocnemius muscle was different in this model than seen within acute HLI models. The extensive muscle necrosis seen within acute models was not reported. The pressure gradients across the collateral arterial beds were low and e-NOS and early growth response-1 genes were not significantly activated to induce collateral artery enlargement (Tang, Chang et al. 2005, Yang, Tang et al. 2008). Gradual constriction through ameroids are thought to better recapitulate the

pathophysiology and atherosclerotic stenosis than acute occlusion (Baffour, Garb et al. 2000, Tang, Chang et al. 2005, Yang, Tang et al. 2008, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). However, ameroid based occlusions do not cause severe ischemia (Dragneva, Korpisalo et al. 2013, Nowak-Sliwinska, Alitalo et al. 2018). Instead, ameroid occlusion results in mild ischaemia to which the muscles adapt effectively compared to acute arterial occlusion (Dragneva, Korpisalo et al. 2013). This suggests that ameroid constriction alone may be inadequate to overcome efficient collateral compensation of blood supply. Ligating and excising the FA after slow onset of ischaemia by ameroids may mitigate compensation of blood supply by collaterals arising from the FA.

1.8 Risk factors in models of HLI

Patients with PAD usually have a number of risk factors including smoking, diabetes, hypertension, older age and dyslipidaemia (Gottsäter 2006, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Few pre-clinical studies have incorporated such risk factors within the animal model employed (Mohamed Omer, Krishna et al. 2016). Atherosclerosis is the leading cause of PAD. Most HLI studies use non-atherosclerotic mice which may exhibit faster recovery to angiogenic therapy during ischaemia. Apolipoprotein E-knockout ($ApoE^{-/-}$) and low density lipoprotein receptor knockout mice are commonly used to simulate the effects of atherosclerosis and dyslipidaemia (Seo, Lombardi et al. 1997, Lotfi, Patel et al. 2013). Notably, $ApoE^{-/-}$ C57BL/6 mice develop significant atherosclerotic lesions, exhibit delayed recovery from ischaemia and respond poorly to angiogenic therapy (Balestrieri, Lu et al. 2010, Dragneva, Korpisalo et al. 2013). Another drawback of using healthy young animals for FA ligation models is that collateral formation occurs rapidly in younger animals in contrast with PAD patients (Madeddu, Emanueli et al. 2006, Mohamed Omer, Krishna et al. 2016). Smoking, diabetes mellitus and hypertension are also major risk factors for PAD and important determinants of recovery from ischaemia in both animals and patients (Waters, Terjung et al. 2004, Dragneva, Korpisalo et al. 2013, Mohamed Omer, Krishna et al. 2016). Angiogenesis, arteriogenesis and recovery of blood supply are diminished in the presence of these risk factors (Waters, Terjung et al. 2004, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). These limitations can potentially be overcome by performing studies within pre-clinical models that incorporate some or all of the risk factors common in patients.

1.9 Differences in mouse strains of HLI

The most common mouse strain used for HLI studies is the C57BL/6 background mouse. Many studies have shown strain-based differences between immunocompetent C57BL/6 and Balb/C background mice, owing to genetic heterogeneity (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). For example, BALB/c mice showed significantly reduced recovery of blood flow after FA ligation compared with C57BL/6 (Scholz, Ziegelhoeffer et al. 2002, Fukino, Sata et al. 2003). The arterial tree structure also differs between the two species, with BALB/c having fewer pre-existing collaterals (Helisch, Wagner et al. 2006, Chalothorn, Clayton et al. 2007, Thomas, Thirumaran et al. 2016). Dokun et al. identified that the quantitative trait loci LSq-1 (loss of tissue after ischaemia) on chromosome 7 of the C57BL/6 mouse strain contributes a protective allele to prevent limb necrosis after induction of HLI (Dokun, Keum et al. 2008). Recently, Sealock et al. carried out congenic mapping to refine the loci on chromosome 7 (*Candq1*) and its candidate genes to create an isogenic strain set with extreme difference in collateral extent to assess their role in ischaemic injury (Sealock, Zhang et al. 2014). The candidate gene identified in the refined loci is now designated as determinant of collateral extent-1 (Dce-1). This study further demonstrated that genetic background-dependent variation in collaterals variation is a major factor underlying differences in ischaemic tissue injury (Sealock, Zhang et al. 2014). In short, among the various strains of common laboratory mice, BALB/c is most susceptible to the development of ischaemia as it is reported to have slower recovery after induction of HLI and is more susceptible to tissue necrosis and limb loss after FA ligation (Helisch, Wagner et al. 2006). This strain may therefore be most appropriate to use in studies of CLI (Krishna, Omer et al. 2016). On the other hand, C57BL/6 mice might be more appropriate as a model of IC (Lotfi, Patel et al. 2013) due to the presence of extensive pre-existing collateral circulation (Limbourg, Korff et al. 2009, Hellingman, Bastiaansen et al. 2010). The severity of tissue necrosis resulting from HLI is reported to vary among mouse strains due to genetic variability. For example, DBA/1J mice have a higher incidence (50%) of necrosis following HLI than C57Bl/6J (20%) or BALB/c (17%) mice (Goto, Fukuyama et al. 2006, Krishna, Omer et al. 2016).

Table I2. Characteristics of pre-clinical studies of HLI using mice which may underlie poor translatability to clinical trials.

Characteristic	Use in pre-clinical studies	Disadvantages
Mouse models	Commonly used models include ligation of FA and ligation and excision model of FA.	Variable ischaemia develops from mild to severe. Most widely used FA ligation and excision model results in natural rapid recovery of blood supply in the ischaemic limb (Tang, Chang et al. 2005, Yang, Tang et al. 2008, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016).
Outcome measures (functional)	Functional endpoints assessments uncommon (when used subjective scoring of limb function usually performed) (Krishna, Omer et al. 2016)	Do not mirror functional end-points in clinical trials which include treadmill testing and six-minute walk test.
Co-morbidities (age, atherosclerosis)	Risk factors and co-morbidities not usually present, young healthy mice are commonly used.	The absence of co-morbidities causes rapid recovery from ischaemia naturally and mice respond effectively to revascularisation therapy (Krishna, Omer et al. 2016).
Strains	Commonly used C57BL/6	C57BL/6 shows rapid recovery from ischaemia due to greater collateral density. BALB/c mice demonstrate significantly less recovery of blood flow after FA ligation compared with C57BL/6 (Krishna, Omer et al. 2016).
Abbreviations: FA; femoral artery		

1.10 Common outcome assessments in HLI studies

1.10.1 Measurement of limb ischaemia

In addition to the type of model employed, a major determinant of the future success of pre-clinical models, in terms of both experimental efficacy and translational value, is the evaluation of outcomes (Leonardo, Robbins et al. 2012).

Most studies of the unilateral HLI model report blood supply within the operated limb in comparison with the un-operated contralateral limb. The most common method used in HLI studies to assess blood flow is laser Doppler perfusion imaging (Greco, Ragucci et al. 2013, Matsui, Watanabe et al. 2017) (LDI). This non-invasive technique is used widely to monitor the effect of various surgical induction methods, drugs or cell therapies on limb blood supply. LDI has been widely used to monitor revascularisation responses after therapeutic interventions within HLI models in mice (Couffinhal, Silver et al. 1998, Lloyd, Yang et al. 2001, Taniyama, Morishita et al. 2001, Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). LDI enables real-time assessment of microcirculation throughout the time frame imaged. LDI technique causes minimal distress to mice and allows non-terminal assessment of physiological superficial blood supply of the limbs. Laser Doppler measures the total local microcirculatory blood supply including the blood supply in capillaries, arterioles, venules and shunting vessels. Laser Doppler detects shifts in the frequency of laser light after it interacts with moving components of tissues such as red blood cells (Humeau, Steenbergen et al. 2007, Limbourg, Korff et al. 2009, Greco, Ragucci et al. 2013, Krishna, Omer et al. 2016). LDI is a non-invasive system that measures flux within tissues. A moving mirror directs reflected laser light from moving red blood cells to a photodetector. Subsequent processing results in a two-dimensional colour-coded image of flux (Limbourg, Korff et al. 2009, Greco, Ragucci et al. 2013). Measurements are expressed in arbitrary Perfusion Units (PU). The unilateral HLI model permits the use of the contra-lateral extremity as a control (Krishna, Omer et al. 2016).

1.10.2 Examination of gross tissue necrosis and limb function

Limb appearance and function after ischaemia induction are assessed in reported studies by observer relative grading methods. Clinical signs manifested due to poor or occluded blood flow to the limb are usually semi-quantitatively assessed by gross examination of the degree of necrosis on the ischaemic limb. A ‘clinical score’ grading based on the number of necrotic toes is suggested to be useful in assessing the recovery from ischaemia achieved by different

therapies and is reported to have low inter-observer variability (Madeddu, Emanueli et al. 2006). The severity of tissue necrosis in the ischaemic limb has been previously assessed using the following scale:

Score	Description
0	No necrosis
1	One toe
2	2 or more toes
3	Foot necrosis
4	Leg necrosis
5	Auto amputation of the leg

(Shireman and Quinones 2005).

A more elaborate scoring system developed by Chalothorn et al. called 'index of ischaemia' or 'foot appearance' ranged from 0 to 11 [0=normal, 1–5=cyanosis or loss of the nail(s), the score representing the number of affected nails; 6–10=partial or complete atrophy of the digit(s), the score reflecting the number of affected digits; 11=partial atrophy of the fore foot (Chalothorn, Clayton et al. 2007).

In a subsequent study, the same authors presented a more simplified 'index of ischaemia' as:

Score	Description
0	Normal
1	cyanosis or loss of the nail(s)
2	partial or complete atrophy of the digit(s)
3	dry necrosis beyond the digits into the front part of foot

(Chalothorn and Faber 2010, Krishna, Omer et al. 2016)

A commonly used functional scoring system for the usage of the hind limb represents an index of muscle function, represented as:

Score	Description
0	Normal
1	No toe flexion
2	No plantar flexion
3	Dragging of the foot

(Chalothorn and Faber 2010).

Chalothorn and Faber, developed another scoring system for muscle function, named 'clinical use score', which grades the ischaemic hind limb usage as:

Score	Description
0	Normal toe and plantar flexion
1	No toe but plantar flexion
2	No toe or plantar flexion
3	Dragging of the foot

(Chalothorn and Faber 2010).

The use of these scoring systems varies in different studies and in many cases the inter-observer variation in assessment is not reported. Furthermore, even though the assessment of the gross appearance of tissue necrosis and functional defects are considered in these types of scoring systems, the scores are subjective and not reported to be conducted by personnel blinded to any intervention being assessed (Krishna, Omer et al. 2016).

1.10.3 Assessment of functional outcomes in HLI models

The quality of life in PAD patients is mainly affected due to exercise related functional limitation (Krishna, Omer et al. 2016). In clinical trials, patients with IC are primarily assessed for improvement in pain-free walking capacity on treadmills or in corridor walking tests (Regensteiner, Gardner et al. 1997, Garg, Tian et al. 2006, Gardner and Afaq 2008), whereas patients with CLI are assessed in terms of limb pain and salvage (Krishna, Omer et al. 2016). Hence, it would be appropriate that PAD pre-clinical animal models incorporate similar outcome measures (Krishna, Omer et al. 2016). Clinically relevant outcome measures in pre-clinical studies may help to better determine interventions for translation to patients.

Treadmill walk testing and six-minute walk testing are used for objective assessment of changes in walking performance in patients with PAD (Hirsch, Haskal et al. 2006, McDermott, Liu et al. 2013, McDermott, Guralnik et al. 2014, McDermott 2015). Functional tests are very rarely performed to assess walking impairment in pre-clinical experiments. It would be appropriate to have similar objective outcome measure to better extrapolate pre-clinical findings of HLI before testing potential interventions in patients (Krishna, Omer et al. 2016). In this regard, treadmill endurance exercise tests have been used in a few pre-clinical studies (Baltgalvis, White et al. 2014, Marcinko, Bujak et al. 2015). It has been suggested that open field tests in mice mirror the six-minute walk test in patients (Tatem, Quinn et al. 2014). In animal models of neuromuscular disease, the open field (OF) test is widely used for assessing

locomotive impairment and could have potential in assessing ambulatory ability in HLI models (Ijomone, Olaibi et al. 2014, Tatem, Quinn et al. 2014).

1.10.4 Response to exercise training in current animal models of HLI

Exercise therapy is an effective non-invasive modality to improve walking in IC patients. The majority of studies in patients that have IC have shown little or no increase in blood flow assessed by ABPI following an exercise programme, even when significant improvements in walking ability have been reported (Stewart , Hiatt et al. 2002, Duscha, Robbins et al. 2011, Meneses, Nam et al. 2018). However, in experimental models of acute HLI, exercise training has been shown to augment recovery of blood supply to ischaemic muscles (Yang, Dinn et al. 1990, Yang, Prior et al. 2008, Cheng, Kuzuya et al. 2010, Rokutanda, Izumiya et al. 2011). Experimental models of HLI mimicking patient response to exercise training would be valuable in better understanding the underlying mechanisms associated with functional improvements to exercise.

Recommendations which have been suggested to develop a clinically relevant mouse model of HLI (Krishna, Omer et al. 2016) are as following:

1) A mouse model of HLI should simulate the pathophysiology of PAD through slow onset of ischaemia that ultimately leads to substantial and ongoing limb ischaemia evidenced by functional impairment;

2) Consideration should be given to incorporation of common risk factors present in patients, such as older age, diabetes or smoking in the mouse model of HLI;

3) Outcome assessments common in human trials which include functional measures should be used for characterising HLI in the mouse model;

4) A mouse model of HLI should demonstrate clinically relevant response to established treatment of PAD such as exercise training.

1.11 Employing a clinically relevant mouse model for assessing a potential treatment

The establishment of a clinically relevant mouse model of HLI with features of PAD facilitates the assessment of potential drugs and the identification of endogenous drug targets for limb ischaemia. Modulating e-NOS activity to improve NO bioavailability has been suggested to be

a potential target for medical therapy. NO is an important modulator of the adaptive response to limb ischaemia in PAD. NO and e-NOS modulating drugs are of great interest for therapeutic revascularisation for limb ischaemia. Evidence suggests that the biguanide drug metformin may have potential for revascularisation in limb ischaemia (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992, Takahashi, Shibata et al. 2015). Metformin is the first line pharmacological therapy used to manage hyperglycaemia in patients with type 2 DM (Saisho 2015). As a generic, low-cost drug, metformin could have an impact in both high and low resource health systems. Metformin has gained popularity recently as a drug with potential to be repurposed for treatment of numerous diseases (NIH 2018). Repurposing of approved drugs for new clinical indications is an area of significant interest (Corsello, Bittker et al. 2017). Drug repurposing is the identification of new therapeutic indications for known drugs. These drugs can either be approved and marketed compounds used daily in a clinical setting, or they can be drugs that have not succeeded in clinical trials and discontinued (Barratt and Frail, 2012). A major advantage of repurposing compounds is that a wealth of pharmacokinetic, pharmacodynamic and toxicological effects have already been determined (Nosengo 2016).

Metformin is well tolerated, and ongoing trials evaluating its use in men without diabetes report low discontinuation rates of approximately 4% (Gillessen, Gilson et al. 2016). Lactic acidosis, the most serious side effect of metformin use in diabetic patients, is very rare and rates are comparable between untreated diabetic patients and those treated with metformin (Salpeter, Greyber et al. 2003). Lactic acidosis is also suggested to be caused by underlying diabetes rather than metformin treatment (Salpeter, Greyber et al. 2003, Gillessen, Gilson et al. 2016).

Metformin has a mild and transient inhibitory effect on the mitochondrial complex 1 of the electron transport chain (Rena, Pearson et al. 2013, Saisho 2015). This induces a drop in cellular energy charge resulting in a fall of the cellular ATP concentration and an increase in both ADP/ATP and AMP/ATP ratios (Rena, Pearson et al. 2013). This activates adenosine monophosphate kinase (AMPKα), a critical energy sensor of cellular energy homeostasis that integrates multiple signalling networks to coordinate a wide array of compensatory, protective, and energy-sparing responses (Viollet, Guigas et al. 2012). Metformin has been demonstrated to lead to phosphorylation of e-NOS resulting in increased NO bioavailability (Takahashi, Shibata et al. 2015). Therefore it is interesting to explore metformin for therapeutic revascularisation in limb ischaemia (Rena, Pearson et al. 2013, Takahashi, Shibata et al. 2015). Small uncontrolled clinical studies have suggested that metformin can improve blood supply in patients with PAD (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992).

Metformin has also been recently reported to improve hind limb blood supply within the acute HLI model by increasing AMPKα and e-NOS activity within ischaemic muscles in order to promote angiogenesis (Takahashi, Shibata et al. 2015).

Activation of AMPKα by metformin has been demonstrated to lead to phosphorylation of e-NOS resulting in increased NO bioavailability, which could subsequently promote revascularisation (Rena, Pearson et al. 2013, Takahashi, Shibata et al. 2015). It has been previously reported that metformin does not promote recovery of blood supply in e-NOS deficient mice in which acute HLI is induced (Takahashi et al., 2015). These findings strongly suggests e-NOS is a key mediator of the action of metformin on limb reperfusion (Takahashi, Shibata et al. 2015).

Additional proposed mechanisms which could improve microcirculatory function by metformin include limiting oxidative stress and favouring mitochondrial biogenesis (Hamburg and Balady 2011) (Steven, Daiber et al. 2017). Endothelial cell migration and proliferation are key processes in revascularisation (Goveia, Stapor et al. 2014). The endothelial-driven proliferative response requires upregulation of mitochondrial mass and function (Tang, Luo et al. 2014). Such mitochondrial biogenesis requires the coordinated replication and expression of mitochondrial DNA with the parallel expression of nuclear-encoded mitochondrial genes (Uittenbogaard and Chiaramello 2014). A key regulator of this process is PGC-1α (Uittenbogaard and Chiaramello 2014). PGC-1α promotes acceleration of angiogenesis by ECs through mitochondrial biogenesis which meets EC metabolic demands during proliferation (Rowe, Jiang et al. 2010). Metformin has been previously reported to upregulate PGC1α in skeletal muscles through AMPKα stimulation (Jager, Handschin et al. 2007, Fernandez-Marcos and Auwerx 2011).

Oxidative stress impairs endothelial function and is associated with impairment of revascularisation in PAD (McDermott 2015). Thioredoxin is an antioxidant protein which regulates the redox response to oxidative stress by maintain the reducing environment of tissues (Nishiyama, Matsui et al. 1999). Metformin has also been reported to attenuate oxidative stress by, for example, downregulating expression of thioredoxin interacting protein (TXNIP) which has been suggested to be a critical protein in promoting severe limb ischemia in diabetes (Chong, Chan et al. 2014, Zhang, Pang et al. 2015). Oxidative stress and mitochondrial biogenesis have been previously shown to be associated with muscle changes relevant for functional improvements resulting from limb ischaemia. TXNIP is a negative regulator of TRX

and has been shown to have adverse effects on ischemia and oxidative stress (Dunn, Buckle et al. 2010). TXNIP knockdown has been shown to rescue blood-flow impairment and improve functional recovery in ischaemic hindlimbs (Dunn, Simpson et al. 2014). Normalisation of hyperglycemia-induced TXNIP expression to non-diabetic levels has been reported to rescue diabetes-related impairment of ischemia-mediated angiogenesis (Dunn, Simpson et al. 2014). In addition, TXNIP has been shown to enhance ischemia-reperfusion injury in response to acute hyperglycemia (Yoshioka, Chutkow et al. 2012). TXNIP knockdown is associated with increased e-NOS expression and NO production (Wu, Zheng et al. 2013, Dunn, Simpson et al. 2014). In ischemia/reperfusion injury, TXNIP is associated with harmful consequences by promoting oxidative damage in tissues via its pro-oxidative effects (Lane, Flam et al. 2013). Metformin has been reported to reduce the expression of TXNIP through AMPKα activation in aortic ECs (Li, Kover et al. 2015).

1.12 Employing a clinically relevant mouse model of HLI for assessing a potential target

Endogenous compounds which modulate e-NOS activity could play important roles for improving revascularisation in limb ischaemia (Forte, Conti et al. 2016). Activity of e-NOS could be enhanced through phosphorylation at serine 1177 or e-NOS dimerisation by regulatory proteins (Forte, Conti et al. 2016). In this regard evidence suggests angiotensin converting enzyme 2 (ACE2) could be an important modulator of e-NOS activity (Zhang, Wang et al. 2014). ACE2, a zinc metalloprotease, is an important component of the renin angiotensin system (Santos, Sampaio et al. 2018). ACE2 catalyses the conversion of angiotensin II (Ang II) to angiotensin-(1–7) (Ang-(1–7) (Rabelo, Todiras et al. 2016, Santos, Sampaio et al. 2018). Ang-(1-7) is an endogenous ligand for the G protein-coupled Mas receptor, which is a cell surface receptor that is highly expressed within the vascular system (Rabelo, Alenina et al. 2011). Ang-(1-7) signalling promotes phosphorylation of e-NOS and release of NO via phosphatidylinositol 3-kinase-protein kinase B (PI3K-protein kinase B) and Akt (Sampaio, Souza dos Santos et al. 2007, Dias-Peixoto, Santos et al. 2008). Ang 1–7 promotes angiogenesis and neo-vessel maturation *in vivo* through the Mas1 receptor (Lovren, Pan et al. 2008, Forte, Conti et al. 2016).

Ang II has been reported to promote the uncoupling of e-NOS by depleting regulatory co-factors (HSP-90 and BH4) required for e-NOS dimerisation, thereby increasing superoxide generation and limiting NO bioavailability (Lin, Lin et al. 2004). Ang II mediates

effects *via* complex intracellular signalling pathways that are stimulated after binding to its main G-protein-coupled cell-surface receptor receptor Ang II type 1 (AT_1R). Ang II, *via* AT_1R, induces phosphorylation of multiple tyrosine kinases, including c-Src, Janus family kinases (JAK), focal adhesion kinase, protein tyrosine kinase 2, p130Cas, and PI3K (Nguyen Dinh Cat, Montezano et al. 2013). Ang II signalling promotes nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity, a major source of ROS in the vasculature (Montezano, Nguyen Dinh Cat et al. 2014) (Santillo, Colantuoni et al. 2015). NOX activation increases ROS which oxidise and depletes regulatory co-factors such as H_4B to induce H_4B deficiency (Nguyen Dinh Cat, Montezano et al. 2013, Li, Youn et al. 2015). Depletion of regulatory co-factor availability results in uncoupling of e-NOS, increase in superoxide production and reduction of NO bioavailability (Santillo, Colantuoni et al. 2015).

Blocking Ang II signalling through Ang II receptor type 1 blockers (candesartan) or ACE inhibitor captopril markedly attenuated e-NOS derived superoxide production while augmenting NO bioavailability, implicating coupling of e-NOS (Oak and Cai 2007). The action of ACE2 in degrading Ang II can limit uncoupling of e-NOS and promote NO bioavailability and limit superoxide generation (Patel and Schultz 2013).

Genetic deficiency of ACE2 ($ACE2^{-/y}$) results in reduced tissue and circulating levels of Ang 1–7 and reduced capacity to degrade Ang II (Thomas, Pickering et al. 2010, Bernardi, Burns et al. 2012, Tikellis, Pickering et al. 2012). $ACE2^{-/y}$ mice have also been reported to demonstrate drastic reduction in e-NOS expression at both protein and mRNA levels, and a decrease in NO concentrations (Yamamoto, Ohishi et al. 2006, Rabelo, Todiras et al. 2016). By promoting Ang 1-7 synthesis and attenuation of Ang II signalling, ACE2 could modulate e-NOS through augmenting e-NOS phosphorylation and limiting e-NOS uncoupling in order to improve NO bioavailability (Sampaio, Souza dos Santos et al. 2007, Patel and Schultz 2013). Thus, ACE2 can promote e-NOS activity to play an important role in limb ischaemia. Despite evidence suggesting potential of ACE2 to play an important role in modulating e-NOS and NO levels, the role of ACE2 in limb ischaemia is unknown. ACE2 locates on the X chromosome in human genome (Zhang, Cong et al. 2018). ACE2 activity have been suggested to be differentially regulated in males and females (Soler, Riera et al. 2012, Soro-Paavonen, Gordin et al. 2012) with serum ACE2 activity higher in males than females (Úri, Fagyas et al. 2016). This suggests that ACE2 might differentially modulate e-NOS in males and females and the role of ACE2 in limb ischaemia may be sex dependent.

1.13 General summary and thesis aims

PAD is an atherosclerotic disease of the lower limb arteries which results in impaired blood supply to the lower limbs. Effective pharmacological treatments to restore blood supply and treat the leg symptoms of PAD are an unmet medical need (Vemulapalli, Dolor et al. 2015). There is urgent need for novel treatments to improve limb blood supply and function in PAD patients unlikely to benefit from existing treatments such as surgical revascularisation, structured exercise programs and cilostozol. Novel experimental treatments which have shown promising efficacy in promoting blood flow in pre-clinical animal models have not translated to effective treatments in clinical trials (Lederman, Mendelsohn et al. 2002, Rajagopalan, Mohler et al. 2003, Olea, Vera Janavel et al. 2009, Belch, Hiatt et al. 2011, Annex 2013, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Moxon et al. 2015, Mohamed Omer, Krishna et al. 2016, McDermott, Ferrucci et al. 2017). This is likely attributable to pre-clinical study characteristics including the reliability of animal model used, study design and endpoints assessed. Recommendations for a mouse model to be clinically relevant in order to better translate findings include: 1) simulating the pathophysiology of PAD through chronic occlusions causing severe ischaemia and functional impairment; 2) incorporating more representative pathology, such as concurrent atherosclerosis; 3) incorporating common risk factors present in patients, such as older age, diabetes or smoking; 4) using outcome assessments common in human trials such as treadmill testing; and 5) demonstrating clinically relevant response to established treatments of PAD such as exercise training (Krishna, Omer et al. 2016).

The development of a clinically relevant mouse model facilitates relevant interpretation and translation of potential interventions and identification of endogenous therapeutic targets. NO mainly synthesised by e-NOS plays an important role in modulating adaptive revascularisation to ischaemic tissues. Repurposing existing treatments for modulating e-NOS to improve revascularisation in PAD and identifying potential endogenous modulators of e-NOS activity in limb ischaemia may be important for the development of effective treatments for PAD. NO is an important promoter of angiogenesis and arteriogenesis for post-ischaemic recovery and NO is primarily derived from e-NOS activity (Yu, deMuinck et al. 2005). Endogenous compounds which modulate e-NOS activity could play important roles in the recovery of blood supply to limb ischaemia and e-NOS could be a potential target for drug development in PAD (Forte, Conti et al. 2016). Activity of e-NOS to enhance NO bioavailability could be promoted

through phosphorylation at serine 1177 and by limiting e-NOS uncoupling (Forte, Conti et al. 2016).

Evidence suggests the drug metformin may have potential to be repurposed to improve blood supply to limb ischaemia (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992) by modulating e-NOS activity (Krishna, Omer et al. 2016).

Evidence suggests angiotensin converting enzyme 2 (ACE2) could be an important endogenous modulator of e-NOS activity (Zhang, Wang et al. 2014) and may play an important role in limb ischaemia.

This thesis is comprised of 3 main experimental sections (chapters 3-5). The general goal of each section was:

1) To develop a novel clinically relevant mouse model of HLI
2) To examine the effect of metformin on HLI in the new mouse model
3) To examine ACE2 deficiency on HLI in the new mouse model.

Chapter 2
General materials and methods

2.1 Ethics and mouse husbandry

The studies in this thesis were performed following ARRIVE guidelines (Kilkenny, Browne et al. 2010) and institutional ethics approvals were obtained for all animal experiments from James Cook University before the commencement of studies. All animal protocols conformed to the Guide for the care and use of Laboratory Animals by the United States National Institutes of Health and the Australian code of Practice for the Care and Use of Animals for Scientific Purpose (7th Edition, 2004). (Ethics approvals are attached in the Appendix)

Mice were sourced from different rodent resource facilities as specified in methods of each chapter. All the Apolipoprotein E null (*ApoE*$^{-/-}$) mice (C57BL/6J background) were obtained from the Animal Resource Centre, Canning Vale, Western Australia. All mice were housed in individual cages and were acclimatised to the Animal Facility at the College of Medicine, James Cook University for one week prior to the experiments. All experimental animals were individually housed in Techniplast cages under a 12:12 hr light-dark cycle (relative humidity: 55-60%; temperature:22±1°C) and provided with standard rodent chow and water *ad libitum* throughout the experiment period.

2.2 Two-stage induction of HLI

Blood supply in the most commonly used model of HLI (the acute ligation and excision of the FA) naturally recovers as a result of shear stress promoting angiogenesis and arteriogenesis (Aranguren, Verfaillie et al. 2009, Lotfi, Patel et al. 2013, Mohamed Omer, Krishna et al. 2016). The slow onset of ischaemia has been attempted with ameroid constrictors and ameroids have been suggested to be fully constricted within 14 days (Baffour, Garb et al. 2000, Tang, Chang et al. 2005, Yang, Tang et al. 2008). However, only mild ischaemia is achieved with ameroid contriction alone (Yang, Tang et al. 2008). This suggests that ameroid constriction alone may be inadequate to overcome efficient collateral compensation of blood supply. Ligating and excising the FA after slow onset of ischaemia by ameroids, may be a strategy to mitigate collateral blood supply compensation arising from the FA. This may result in severe ongoing ischaemia. Therefore, in this novel two-stage method of HLI induction, a slow onset of ischaemia achieved by ameroid constriction, followed by ligation and excision of the FA to induce onset of severe ischaemia was proposed.

HLI was induced in the left hindlimb of each mouse (Figure C2.1). Mice were placed under general anaesthesia using isoflurane (2.0% induction dose and 1.8% maintenance dose plus oxygen at 1 L/s). Surgery was performed at 20x magnification under a stereotactic microscope. The first operation involved exposing the left femoral bundle through a vertical 0.5–1 cm skin incision above the inguinal ligament. The femoral vein and nerve were carefully dissected free from the artery. Two custom made ameroid constrictors of 0.25mm internal diameter (Research Instruments SW, Escondido, California, US) were placed on the FA, one immediately distal to the inguinal ligament and one proximal to the sapheno-popliteal bifurcation (Figure C2.1B). The incision was closed using absorbable polygalactin sutures (Ethicon) and the mouse was monitored until recovery. Ameroids have been suggested to be fully constricted within 14 days (Tang, Chang et al. 2005, Yang, Tang et al. 2008). However, only mild ischaemia is achieved with ameroids alone compared to the severity of ischaemia achieved with FA excision (Yang, Tang et al. 2008). Therefore, in this novel 2 stage method of HLI induction, 14 days after ameroid constriction, a second surgery was performed by making a longitudinal incision above the inguinal ligament to expose the FA which was ligated with 6-0 silk sutures (Ethicon) external to the location of the ameroid constrictors and the intervening segment was excised with the ameroids (Figure C2.1C). The incision was closed using 6-0 absorbable polygalactin sutures (Ethicon) and the animals were monitored until recovery.

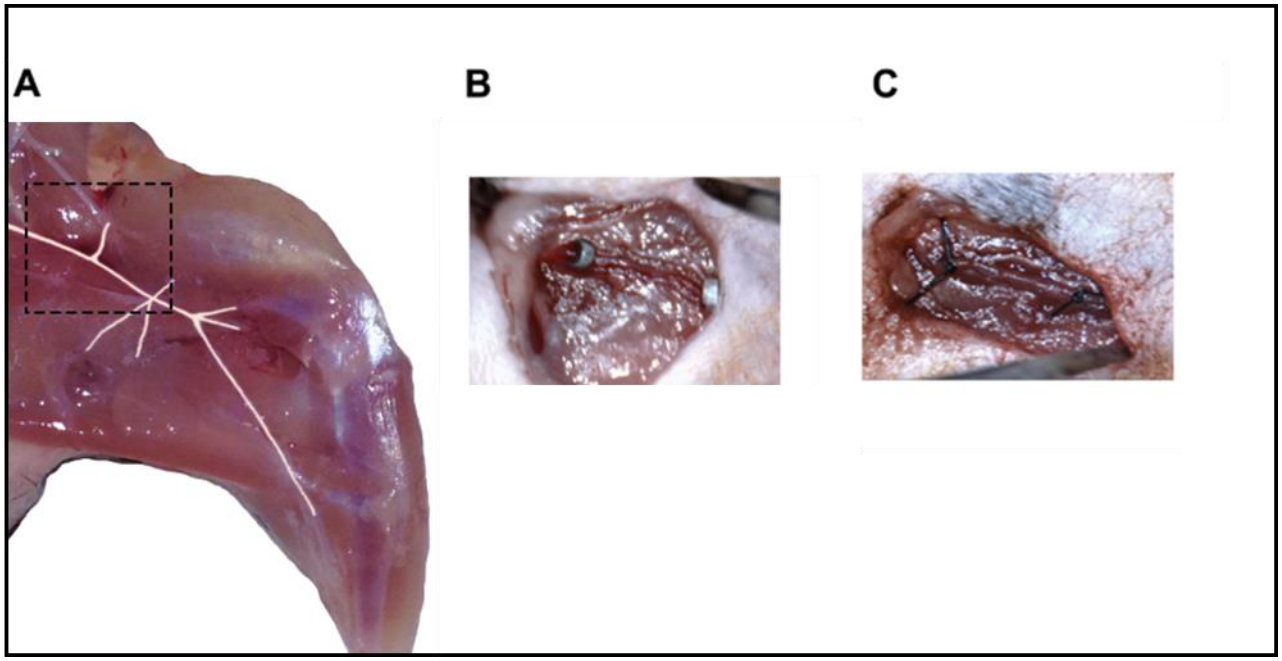


Figure C2.1 The two-stage surgical HLI model. A, shows the region where the surgical manipulations of FA were performed. **B**, ameroids placed on proximal and distal ends of FA and **C**, ligated and excised FA.

2.3 Laser Doppler imaging

LDI was performed to non-invasively monitor blood supply of hind limbs in mice. LDI measurements were carried out at different time points stated in specific chapters. LDI was carried out with a high-resolution laser Doppler perfusion imager (LDPI; Moor Instruments, UK). Hair was removed from the limbs using depilating cream on the day before LDPI measurements. The mouse was placed under general anaesthesia with isoflurane (2.0% induction dose and 1.8% maintenance dose plus oxygen at 1 L/s) and maintained at 37ºC using a heated pad. During imaging, ambient light and temperature was carefully controlled to avoid background variations in LDI measurements. The LDI source was mounted 26 cm above the mouse limbs. The scanning produced a color-coded image and the image analysis software (Laser Doppler Perfusion Measure, V3.08, Moor Instruments, UK) was used to calculate the limb mean flux units, which represents a quantitative analysis of tissue perfusion on a scale of 0 to 1000. Pixels in the acquired image reflected the blood flow value, referred to as a perfusion unit (flux unit). To account for variations in ambient light, temperature, and other conditions, perfusion units were presented as the ratio of ischaemic (left) to non-ischaemic (right) measurements in the same mouse (flux ratio).

2.3.1 LDI repeatability

The repeatability of LDI image measurements were assessed using 12 *ApoE*$^{-/-}$ mice aged 12 weeks without HLI. LDI imaging was carried out as described above. Hind limbs were selected manually as the region of interest for LDI image analysis and the ratio of left to right mean flux values was obtained for each mouse. Measurements were carried out independently by two investigators to determine inter-observer repeatability. Intra-observer repeatability was performed by one investigator who carried out 3 measurements on the same image to determine intra-observer repeatability. Acceptable repeatability measures were suggested for the inter-observer repeatability of LDI measurement (coefficient of variance=4.49%) and the intra-observer repeatability of LDI measurement (coefficient of variation=4.17%) (shown in the tables below).

Table C2.1. Inter-observer repeatability results of LDI measurements

Mouse ID	Observer 1	Observer 2	Mean	Standard deviation
1	0.81	0.78	0.79	0.016
2	1.52	1.46	1.49	0.04
3	1.05	0.97	1.01	0.05

4	1.24	1.33	1.29	0.06
5	1.01	0.96	0.99	0.03
6	0.82	0.86	0.84	0.02
7	0.97	1.07	1.02	0.07
8	0.94	0.95	0.94	0.006
9	0.94	0.91	0.92	0.02
10	0.89	0.92	0.90	0.01
11	0.97	0.97	0.97	0.003
12	0.92	1.17	1.05	0.18
		Mean	1.023032	0.04603
		CoV %	**4.49936**	
		ICC	**0.942**	
		95% CI	**0.79;0.98**	
		95% LOA	**-20.75;15.85**	

Abbreviations: CoV; coefficient of variation, ICC; inter-class coefficient of variation, CI; confidence interval, LOA; limits of agreement

Table C2.2: Intra-observer repeatability results of LDI measurements

Mouse ID	Reading 1	Reading2	Reading 3	Mean	Standard deviation
1	0.78	0.78	0.75	0.78	0.023
2	1.46	1.54	1.40	1.48	0.064
3	0.97	0.96	0.95	0.98	0.045
4	1.33	1.24	1.24	1.26	0.046
5	0.96	1.03	1.06	1.02	0.041
6	0.86	0.82	0.84	0.83	0.020
7	1.07	0.94	0.95	0.98	0.062
8	0.95	0.91	0.92	0.93	0.016
9	0.91	0.86	0.85	0.89	0.040
10	0.92	0.92	0.92	0.91	0.012
11	0.97	0.93	0.94	0.95	0.020
12	1.17	1.13	1.07	1.07	0.111
			Mean	1.01	0.042
			CoV %	**4.17**	
			ICC	**0.987**	
			95% CI	**0.965;0.996**	
			95% LOA	**-0.09;0.14**	

Abbreviations: CoV; coefficient of variation, ICC; inter-class coefficient of variation, CI; confidence interval, LOA; limits of agreement

2.4 Treadmill exercise performance test

Mice were fasted for 2 hours prior to being placed on the treadmill. The mice were acclimatised to the treadmill (Columbus Exeter 3/6, Columbus instruments; Columbus, OH) before experiments commenced (prior to baseline measurements). This was done over 3 consecutive days by placing them on the treadmill for 15 minutes each day at a speed of 10m/minute. If the mice tried to rest during the acclimatisation period they were gently pushed onto the treadmill belt. The treadmill outcome assessments were carried out by placing mice on the treadmill at an initial speed of 10m/min for 5 min to warm up. Speed was maintained at 10m/min and shock grids of 3Hz was kept on until the mouse exhausted. Exhaustion of the mouse was defined if the mouse returns to the shock grid 10 times despite a 3Hz electrical stimulus to encourage walking on the belt and the treadmill software recorded the total distance walked by a mouse until exhaustion. Treadmill testing was carried out at time-points stated in specific chapters.

2.5 Observational assessment of limb function and ischemia

Assessment of limb function and gross limb ischemia were performed according to the descriptors used in previous studies (He, Luo et al. 2006, Westvik, Fitzgerald et al. 2009). Tarlov functional scoring is shown in table C2.3 and ischaemia score and representative figures of descriptors are shown in table C2.4 and figure C2.2.

Table C2.3 Descriptors of Tarlov functional scoring

Score	Description
0	No movement
1	Barely perceptible movement, no weight bearing
2	Frequent and vigorous movement, no weight bearing
3	Supports weight, may take 1 or 2 steps
4	Walks with only mild deficit
5	Normal but slow walking
6	Full and fast walking

Figure C2.2. Descriptors for scoring the appearance of the ischaemic versus non-ischaemic contralateral limb.

Table C2.4 Descriptors of ischemia scoring

Score	Description
0	Auto-amputation of leg
1	Leg necrosis
2	Foot necrosis
3	Two or more toe discoloration
4	One toe discoloration
5	Two or more nail discolorations
6	One nail discoloration
7	No necrosis

2.6 Euthanasia and tissue collection

Mice were euthanised by carbon dioxide asphyxiation at the experimental endpoint. Blood samples were collected from the right ventricles by cardiac puncture with a 1ml syringe fitted to a 20-22G needle. Samples were transferred into heparin coated tubes and placed on ice.

Blood samples were centrifuged for 10 min at 5000g and 5 minutes and 2000g for 10 minutes at 4 °C to separate platelet poor plasma. The separated plasma samples were aliquoted into 1.5 ml microfuge tubes, flash frozen in liquid nitrogen and stored at -80 °C.

Gastrocnemius and gracialis muscles of ischaemic and non-ischaemic limbs of mice were harvested. A portion of each muscle was stored in optimum cutting temperature compound (Tissue-Tek, Australia), flash frozen in liquid nitrogen and stored at -80° C. A portion of the

gastrocnemius muscle and gracialis muscle was stored in RNA later and placed on ice and stored at -20 °C.

2.7 Statistical analysis

Graphpad Prism V6.0 (GraphPad Software, San Diego, CA) and R studio software programs were used to analyse data. Graphpad was used to construct graphs. Sample sizes for each study were estimated as described in the methods of each chapter using G*power software. Data were tested for normality using D'Agostino-Pearson normality test. Data with normal distribution were expressed as mean ± standard error of mean (SEM) and analysed using parametric tests. Non-normally distributed data were expressed as median and interquartile ranges (IQR) and analysed using non-parametric tests. Linear mixed effects (LME) model analysis or 2-way repeated measures ANOVA were used to compare LDI data between groups. For LME analyses variation between individual mice were treated as random effects. LDI data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Where necessary data was log transformed or square root transformed to fit model assumptions. Interaction between time and groups were assessed by LME. Comparison of clinical scores were performed by 2-way repeated measures ANOVA. LME model analysis was used to compare treadmill data between groups. For LME analyses variation between individual mice were treated as random effects. Treadmill data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Where necessary data was log transformed or square root transformed to fit model assumptions. Interaction between time and groups were assessed by LME. In all cases a p value of <0.05 was considered to be statistically significant.

Chapter 3
Development of a new experimental mouse model of lower limb ischaemia

3.1 Introduction

PAD is an atherosclerotic disease of the lower limb arteries which results in impaired blood supply to the lower limbs (Golledge 1997, Norman, Eikelboom et al. 2004, Shu and Santulli 2018). Patients with PAD experience debilitating lower limb symptoms including excruciating leg pain during walking (IC), rest pain, ischaemic ulceration and gangrene of the limb (CLI) which severely limit their physical activity and increase the risk of amputation (McDermott, Greenland et al. 2001, Morris, Rodriguez et al. 2014).

Current treatments aimed at improving limb haemodynamics, improving walking performance and preventing mobility loss in PAD patients include surgical revascularisation, supervised exercise and cilostozol (Thukkani and Kinlay 2015, Meneses, Ritti-Dias et al. 2017, Kithcart and Beckman 2018, Morcos, Louka et al. 2018). These treatments are associated with significant limitations and a growing number of patients are not suitable for current PAD treatments (Lawall, Bramlage et al. 2011). Endovascular or open surgical revascularisation are associated with serious risk of complications and poor long-term effectiveness (Piazza, Squizzato et al. 2015, Rosenfield, Jaff et al. 2015, Tepe, Laird et al. 2015, Wiseman, Fernandes-Taylor et al. 2017). Supervised exercise treatment may not be feasible with the presence of co-morbidities such as old age and are not widely available, possibly because of limited evidence on long-term effects (Regensteiner 2004, Morris, Rodriguez et al. 2014). Cilostozol is the only drug currently approved for treating limb symptoms of PAD, however it may be associated with adverse side effects and cilostozol is not publicly funded in many countries including Australia as the benefit was deemed to be too modest (Bedenis, Stewart et al. 2014, Golledge, Moxon et al. 2018).

Numerous new molecular and cellular therapies have been tested for patients unlikely to benefit from current treatment options. However, treatments tested to be effective in pre-clinical studies to promote revascularisation, restore function and prevent amputation have not successfully translated in clinical trials (Lederman, Mendelsohn et al. 2002, Rajagopalan, Mohler et al. 2003, Olea, Vera Janavel et al. 2009, Belch, Hiatt et al. 2011, Annex 2013, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Moxon et al. 2015, Mohamed Omer, Krishna et al. 2016, McDermott, Ferrucci et al. 2017). Key reasons include the inability of currently used pre-clinical animal models and outcome measures to successfully predict clinical effectiveness (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016, Rigato, Monami et al. 2017).

A major disadvantage of currently used pre-clinical animal models to simulate PAD is the rapid natural recovery of blood supply to ischaemic tissues which occur after HLI induction (Aranguren, Verfaillie et al. 2009, Lotfi, Patel et al. 2013, Mohamed Omer, Krishna et al. 2016). Interventions are assessed for augmenting the speed of natural recovery of blood supply rather than improving ischaemia from a state of ongoing HLI (Mohamed Omer, Krishna et al. 2016). The most commonly used mouse model of HLI is developed through the acute ligation and excision of the FA (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016). This results in sudden changes in fluid shear stress (FSS) within collateral arteries to alter gene expression patterns through shear responsive elements subsequently promoting arteriogenesis and angiogenesis to restore blood supply to the ischaemic tissues (Topper and Gimbrone Jr 1999, Garcia-Cardena, Comander et al. 2001, Tang, Chang et al. 2005). Typical patient presentation of PAD involves ongoing state of ischemia arising from chronic ischaemic insult to the limb in the presence of co-morbidities such as old age and atherosclerosis (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Ameroid constrictors have been used to develop gradual onset of ischaemia in animal models of HLI (Baffour, Garb et al. 2000, Tang, Chang et al. 2005, Yang, Tang et al. 2008). However, ischaemia achieved within the model of ameroids alone was significantly less compared to ischaemia achieved through a ligation and excision of the FA (Tang, Chang et al. 2005). This suggests that ameroid constriction alone may be inadequate to overcome efficient collateral compensation of blood supply. In order develop the onset of severe ischaemia, excising the FA may limit blood supply through collaterals arising from the FA after slow onset of mild ischaemia by the ameroids.

PAD patients typically present with a history of acute exacerbation of chronic symptoms of leg pain on walking and have ongoing ischaemic symptoms (Bonaca, Gutierrez et al. 2016). In addition to the method of occlusion, risk factors of PAD such as aging and atherosclerosis are associated with diminished post-ischaemic recovery and are not commonly incorporated into pre-clinical models of HLI (Faber, Zhang et al. 2011, Dragneva, Korpisalo et al. 2013, Krishna, Moxon et al. 2015). Therefore, a clinically relevant mouse model of HLI is needed incorporating chronic occlusion and risk factors to better mimic the pathophysiology of occlusion and ongoing ischaemia.

Differences in outcome measures used in pre-clinical studies in relation to clinical trials may also be associated with poor translatability of pre-clinical findings. The American Heart Association/American College of Cardiology clinical practice guidelines for PAD recommend both treadmill exercise testing (Class I recommendation, Level of Evidence-B) and six-minute walk testing (Class IIb recommendation, Level of Evidence-B) for objective assessment of changes in walking performance in patients with PAD (Hirsch, Haskal et al. 2006, McDermott 2015). Treadmill exercise testing is the most commonly used objective outcome measure in randomised clinical trials studying interventions to improve walking performance in people with PAD. The six-minute walk test has been well validated in patients with PAD (McDermott, Liu et al. 2013, McDermott, Guralnik et al. 2014, McDermott 2015). Functional tests mirroring clinical trial outcome measures of limb function are lacking in pre-clinical studies and objective functional tests are very rarely performed to assess walking impairment in pre-clinical experiments (Zhong, Eliceiri et al. 2003, Baltgalvis, White et al. 2014). Treadmill endurance exercise tests have been used in a few pre-clinical studies (Zhong, Eliceiri et al. 2003, Baltgalvis, White et al. 2014, Marcinko, Bujak et al. 2015, Deng, Yang et al. 2016, Krishna, Omer et al. 2016). It has been suggested that open field tests in mice mirror the six-minute walk test in patients (Tatem, Quinn et al. 2014). It would be appropriate to have similar objective outcome measures to better extrapolate pre-clinical findings of HLI before testing potential interventions in patients. The treadmill test and open field test need to be explored for examining functional impairment during HLI.

The response to exercise training in current pre-clinical HLI models differs from the response in PAD patients. Management of patients by supervised treadmill exercise and home-based exercise that incorporate behavioural change techniques significantly improve pain-free and maximal walking distance in people with PAD (McDermott, Ades et al. 2009, Gardner, Parker et al. 2011, Hamburg and Balady 2011, Fakhry, van de Luijtgaarden et al. 2012, McDermott, Liu et al. 2013, Gardner, Parker et al. 2014, McDermott, Ferrucci et al. 2017, McDermott 2018). Studies in PAD patients, however indicate that the functional benefits of exercise are not a result of increased gross blood supply to ischaemic limbs (Stewart , Hiatt et al. 2002, Parmenter, Raymond et al. 2010, Meneses, Ritti-Dias et al. 2017). In experimental models of acute HLI, exercise training has been shown to augment recovery of blood supply to ischaemic muscles (Yang, Dinn et al. 1990, Yang, Prior et al. 2008, Cheng, Kuzuya et al. 2010, Rokutanda, Izumiya et al. 2011). This suggests that findings of functional changes observed in current HLI models may not be reliable for translation. Models of HLI require validation of

response to established interventions in PAD patients such as exercise training to be reliable in predicting clinical outcomes.

The hypotheses in this chapter were:

Primary: A novel two-stage induction of HLI through slow onset of mild ischaemia induction by ameroids on the FA followed by induction of severe ischaemia by excision of the FA would produce more severe ongoing HLI over the experimental period of 4 weeks.

Secondary:

1) Functional impairment as a result of ongoing ischaemia will be greater in mice with two-stage HLI than mice with acute HLI.
2) Exercise training will improve treadmill exercise performance in mice with two-stage HLI independent of changes to limb blood supply.

The aims in this chapter were:

Primary: To develop a new mouse model of ongoing severe HLI through a slow onset two-stage method of HLI induction.

Secondary:

1) To examine functional impairment in the two-stage model and acute HLI model by treadmill test and open field test.
2) To examine the effect of exercise training on functional capacity and limb blood supply in the two-stage model of HLI.

3.2 Methods

3.2.1 Ethics approvals and mouse husbandry

Institutional ethics was obtained for the studies presented in this Chapter (Appendix) and mice were maintained in individually housed Techniplast cages under a 12:12 hr light-dark cycle (relative humidity: 55-60%; temperature:22±1°C) and provided with standard rodent chow and water *ad libitum* throughout the experiment period as described in Chapter 2.1.

3.2.2 Study design 1: To establish a new mouse model of two-stage HLI

Four groups of male *ApoE*$^{-/-}$ mice aged 12 months were used in this study as follows, Group 1= acute HLI model (n=10), Group 2= acute sham (n=8), Group 3= two-stage HLI model (n=10) and Group 4= two-stage HLI sham (n=8). Respective surgeries were performed for each group as described below and blood supply was assessed by LDI as the primary outcome at different time-points as illustrated in Fig M3.0.

3.2.2a Sample size

The sample size was estimated for study design 1 using the primary outcome measure of LDI. The expected LDI outcome for the two-stage HLI model was calculated using LDI data from a previous study which used the acute PAD model (Yang, Tang et al. 2008). In that study, 14 mice were imaged 28 days after acute ischaemia induction and mean (SD) flux ratio was 0.76 (0.15). It was assumed that the expected LDI outcome in the two-stage model group will be 30% less than the acute HLI group over 4 weeks after ischaemia induction. This is because a previous study has suggested blood supply decrease by 30% leads to functional impairment (Brenes, Jadlowiec et al. 2012). Based on these assumptions and aiming to achieve an 80% power with an alpha of 0.05 it was estimated that 9 mice per HLI group were needed.

3.2.2b HLI induction

3.2.2b1: Induction of two-stage HLI model

Unilateral two-stage HLI was induced in the left hindlimbs of male *ApoE*$^{-/-}$ mice (n=10) mice by the 2 step method as described in Chapter 2. The respective shams received similar surgeries in which the FA was dissected free but not occluded. The acute HLI was induced in the left hindlimbs of mice as described below.

3.2.2b2: Induction of acute HLI

Male *ApoE*$^{-/-}$ mice (n=10) were subject to the induction of ischaemia through the acute HLI method. Mice were anaesthetised with isoflurane and maintained under isoflurane anaesthesia at a flow rate of 1L/s during the surgery. The left FA was exposed through an approximately 0.5–1 cm vertical skin incision adjacent to the inguinal ligament. The femoral artery was separated from the femoral nerve and vein and ligated with 6-0 silk sutures (Ethicon, Johnson and Johnson, Melbourne Australia) distal to the inguinal ligament and proximal to the popliteal bifurcation site. The FA between the ligatures was excised, the surgical site was closed using 4-0 vicryl sutures (Ethicon) and disinfected with Betadine (BETADINE, Australia). A similar surgery without manipulation was performed on sham controls in which the FA was dissected free but not ligated. Following the surgery, mice were removed from isoflurane anaesthesia and monitored carefully until conscious and subsequently returned to cages.

3.2.2c: Laser Doppler Imaging:

LDI was performed as described in Chapter 2 at several time-points (Figure M3.1) within the experimental period at baseline and weekly after ischaemia induction.

3.2.2d Observational functional scoring and assessment of ischaemia

Observational assessment of limb function (Tarlov score) and ischaemia (Ischaemia score) were carried out at the same time-points as LDI assessments. Scoring was performed as detailed in Chapter 2.

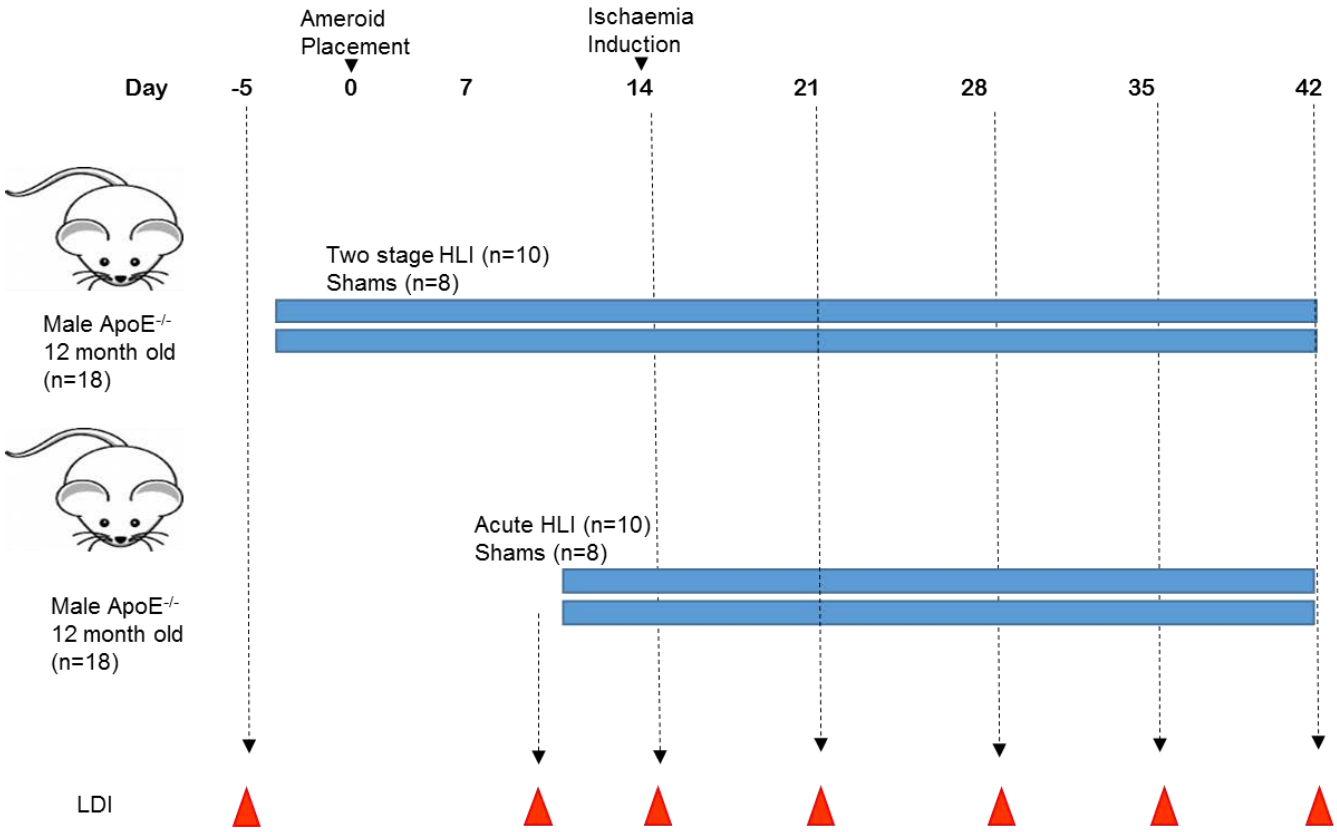


Figure M3.1: Study design to examine the effect of two-stage HLI induction on limb blood supply. Male *ApoE*$^{-/-}$ mice were subjected to two-stage HLI or acute HLI with their respective shams. LDI was performed at baseline and 1 week after ameroid placement in the two-stage HLI and respective sham groups. Acute HLI and the respective sham group were monitored with LDI at baseline and all groups were monitored immediately after ischaemia induction and at weekly intervals over 4 weeks after ischaemia induction.

3.2.3 Study design 2: To assess limb function in HLI models by treadmill and open field tests

Evaluation of the treadmill test and open field test for the assessment of functional impairment of hindlimbs were performed on male *ApoE*$^{-/-}$ mice (n=23) aged 12 months. Mice were separated into four groups; the two-stage HLI (n=6), acute (n=6), acute sham (n=5) and two-stage sham (n-=6). HLI was induced in respective groups as in Study 1.

3.2.3a Treadmill test

The treadmill test was performed on the mice as described in Chapter 2. The treadmill tests were performed at several time-points within the experiment as shown in Figures M3.2 and M3.3.

3.2.3b Open field test

Mice were acclimatised to a sound-attenuated testing room 2 hours prior to the test. The mice were fasted during the acclimatisation period with *ad libitum* access to water under normal lighting. The open field (OF) was a square box (42 cm×42 cm×42 cm) made of opaque plastic. For assessment, each mouse was placed in the centre of the arena and video recorded using a camera (Logitech) supported with acquisition software (*Capture Star Ver. 1*; CleverSys Inc) for 20 minutes. At the end of each test, the surfaces of the OF box were cleaned with 70% ethanol and dried. This was in order to prevent behavioural influence in the subsequent mouse, which may result from cues (such as body odour, urine, faeces, hair and sweat) from the previously tested mouse. All experimental procedures were conducted during the light phase of the cycle, between 8:00 a.m. and 3:00 p.m. Behavioural test room lighting, temperature, and noise levels were kept consistent for all subjects. The acquired recordings were analysed by the TopScan Lite (*High throughput version 2.0*; CleverSys Inc).

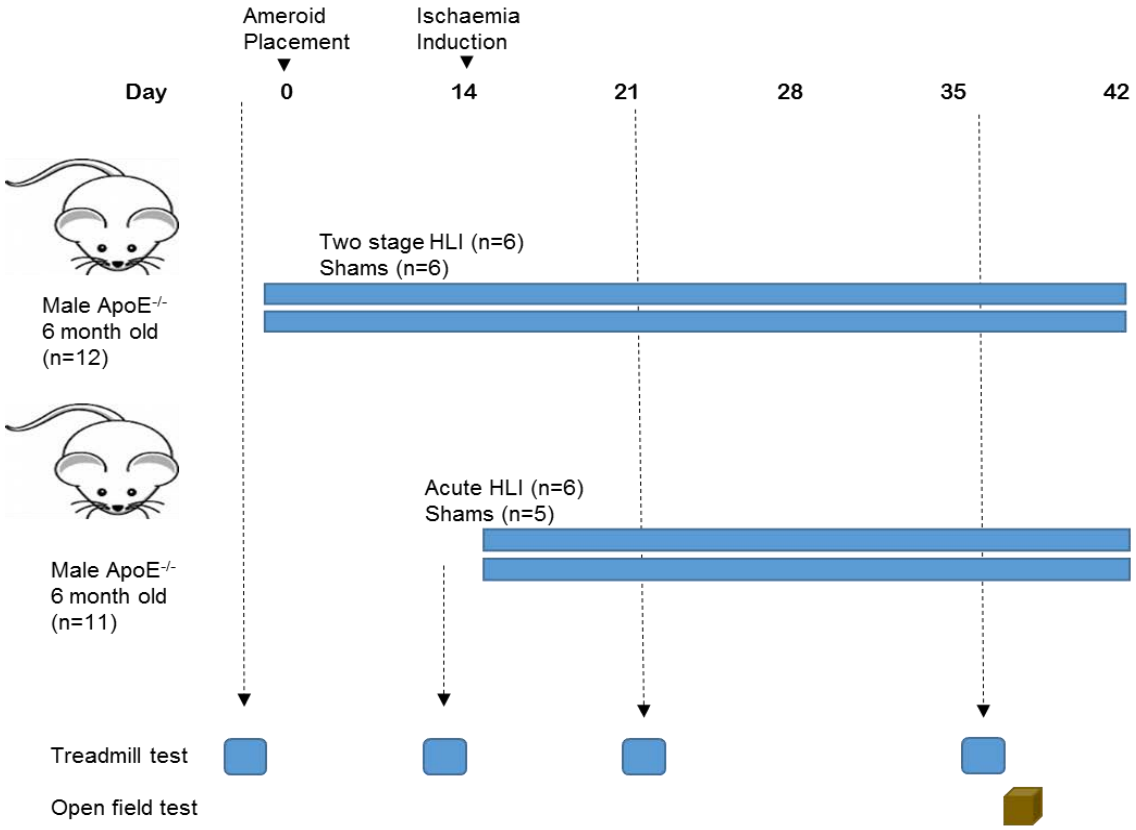


Figure M3.2: Study design to assess limb function in HLI models by the treadmill test and open field test. Treadmill tests were performed at baseline, 2 days after ischaemia induction and at days 23 and 37 of the experimental period in all groups. The open field test was conducted on day 38 of the experimental period.

3.2.4 Study design 3: To examine the effect of exercise training on the two-stage model of HLI

Male *ApoE*$^{-/-}$ mice aged 6 months (n=30) were used for this study. HLI was established via the two-stage HLI induction method described in Chapter 2.2. After 5 days from HLI induction, mice were separated to two groups, one group of mice (n=15) received exercise training on a running wheel and the control group had no access to the running wheel. Mice in the exercise group received 180 to 200m (between 30-45 minutes of wheel access) of exercise each day in a running wheel (8 Station Home Cage Running Wheel System, Columbus Instruments) over 4 weeks. Each mouse was placed on a running wheel within a small chamber which prevented the mouse from escaping the running wheel during the exercise period and left to run on the wheel. The wheel counts were monitored on the running wheel software until they reached 600 counts and the mice was removed from the wheel and returned to its cage. The control mice

did not receive access to the running wheel at any point of the experiment. Primary outcome measures were LDI to assess blood supply and treadmill exercise test to assess function. These were performed as described in Chapter 2.3 and Chapter 2.4, respectively within the experimental period (Fig M3.3). During the assessment and analysis of the outcome measures of LDI and treadmill performance, the group to which each mouse belonged was blinded to the investigator. This was done by allocating a random label against each cage and keeping a strict record of the allocation during each assessment time-point. After the assessment mice were allocated their original number.

3.2.5 Statistical analysis

Graphpad Prism V6.0 (GraphPad Software, San Diego, CA) and R studio software programs were used to analyse data. Graphpad was used to construct graphs. Data were tested for normality using D'Agostino-Pearson normality test. Data with normal distribution were expressed as mean ± standard error of mean (SEM) and analysed using parametric tests. Non-normally distributed data were expressed as median, interquartile ranges (IQR) and analysed using non-parametric tests. In study 1 and study 2, comparisons between groups for the LDI data, modified ischaemia score data, Tarlov score data and treadmill walking distance data were carried out using 2-way ANOVA analysis. In study 2, data were compared between groups with Mann Whitney U test for the endpoint treadmill walking distance data and open field test walking distance data. In study 3, LME was used to compare LDI data between groups. For LME analyses variation between individual mice were treated as random effects. LDI data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Interaction between time and groups were assessed by LME. LME model analysis was also used to compare treadmill data between groups. For LME analyses variation between individual mice were treated as random effects. Treadmill data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Data were square root transformed to fit model assumptions. Interaction between time and groups were assessed by LME. In all cases a p value of <0.05 was considered to be statistically significant.

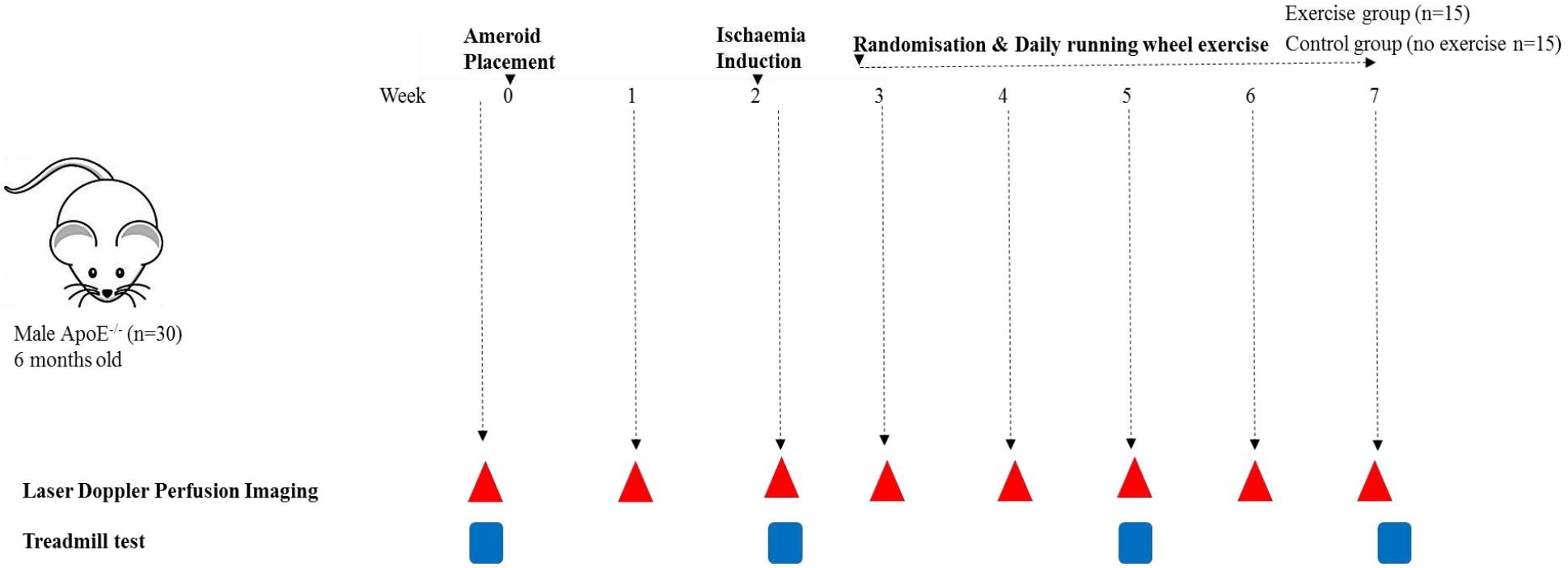


Figure M3.3: Study design to examine the effect of exercise training in the two-stage model of HLI. Mice subject to two-stage HLI were randomised and started on exercise training on a running wheel 5 days after ischaemia induction. LDI was performed at baseline and days 7, 16, 21, 28, 35,42 and 49. The treadmill tests were performed at baseline, days 16 and 35 and 48.

3.3 Results

3.3.1: Mice with two-stage HLI showed more severe and prolonged ischemia than mice with acute HLI induction

Blood supply in the hindlimbs of mice were similar among all experimental groups before surgery (Figure R3,1). Ameroid constriction for 2 weeks in the two-stage HLI group before excising the FA did not significantly decrease blood supply to the limbs compared to blood supply before placing ameroids ($p=0.481$; Figure R3.1C). Immediately after excision of the FA in the 2 stage HLI group, the blood supply was significantly lower ($p<0.001$) compared to blood supply before excision and after 2 weeks of ameroid constriction producing a severe state of ischaemia (Figure R3.1C).

The excision of the FA in the acute HLI group resulted in a comparable level of HLI to the 2 stage HLI group immediately after HLI induction (Figure R3.1). In the 4 weeks after surgery, HLI was significantly more severe ($p=0.036$) in the two-stage HLI model compared to the acute HLI model (Figure R3.1B).

The respective shams of each group, the acute HLI shams and the two-stage HLI shams, did not show differences in limb blood supply between baseline () and experiment endpoint (Figure R3.1B). Limb ischaemia was significantly greater in the HLI groups compared to their respective sham groups throughout the experiment ($p<0.001$; Figure R3.1B).

Gross examination of the severity of tissue damage in the ischaemic limb assessed by ischaemia scoring showed at the start of the experiment ischaemia scores were similar in all mice. The ischaemia scoring system showed that the two-stage HLI group had significantly greater limb ischaemia than mice in the acute HLI group ($p=0.012$=, Figure R3.2A) after ischaemia induction until the experimental endpoint.

3.3.2: Functional impairment assessed by scoring was greater in the two-stage HLI mice than mice with the acute HLI

Hindlimb function assessed by Tarlov scoring showed that at the start of the experiment all mice had no functional impairment (score=7). After ischaemia induction by FA excision, two-stage HLI mice had significantly greater functional impairment than mice in the acute HLI group ($p<0.001$, Figure R3.2B).

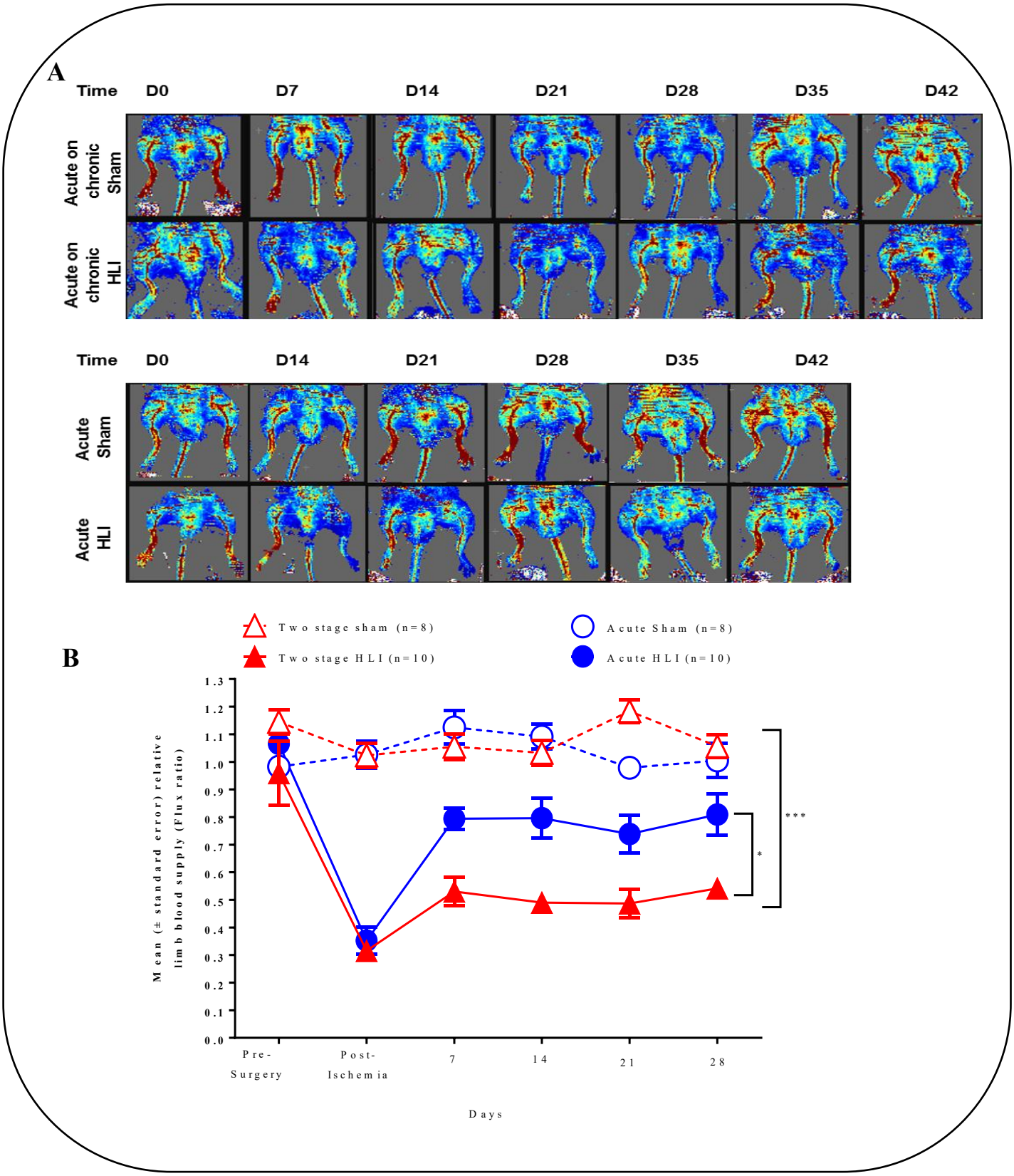


Figure R3.1: Comparison of limb blood supply in the two mouse models of HLI and their respective shams. A: Representative LDI images of the different groups monitored at several timepoints. B: LDI perfusion measurements between the acute and two-stage HLI models and their

respective sham controls. All data expressed as mean±SEM. Comparisons between groups were performed by 2-way ANOVA and significant differences $p<0.05$ indicated by *, $p<0.001$ ***.

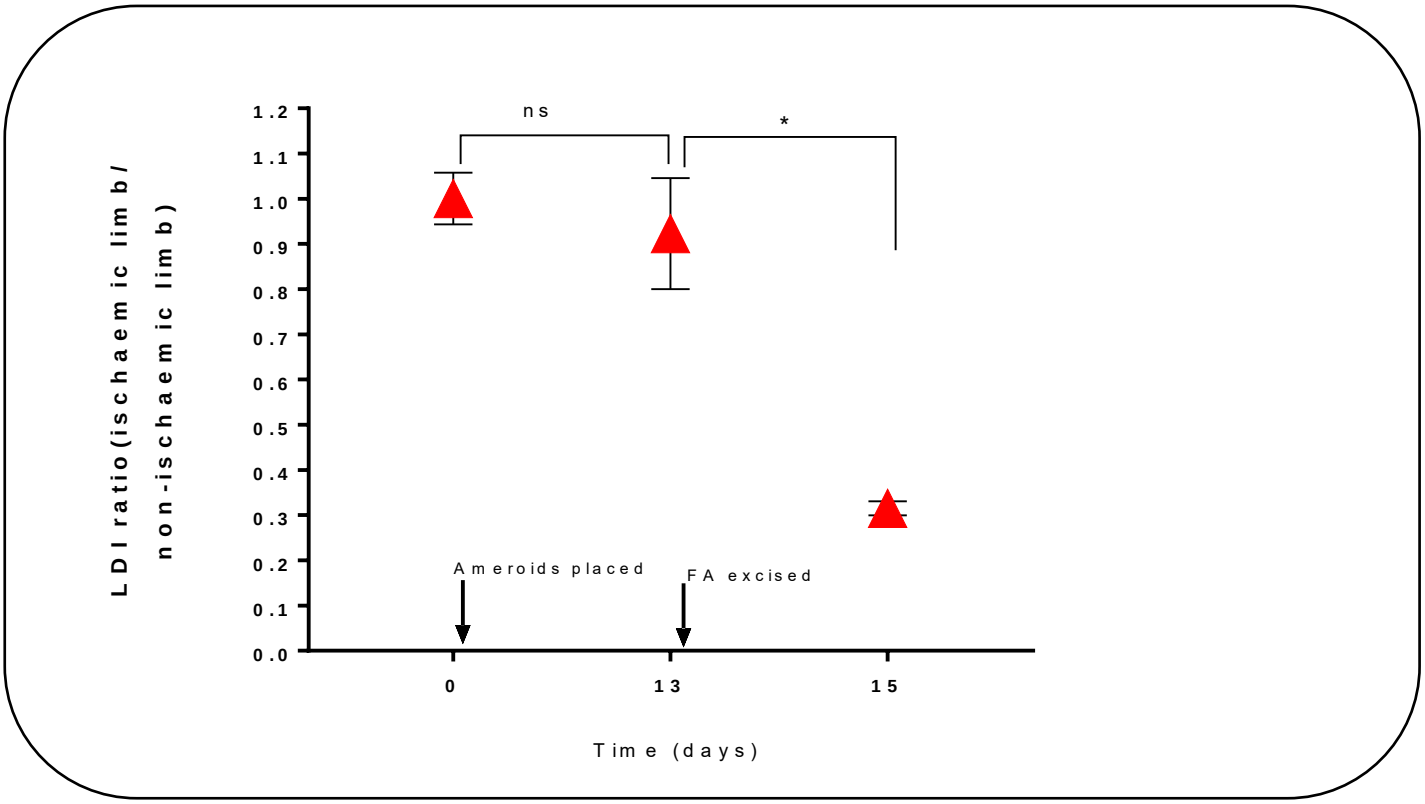


Figure 3.1C. Changes in limb blood supply during the two-stage HLI procedure in mice (n=10). LDI measurements at baseline (without ameroids), 2 weeks after ameroid placement and immediately after excision. Data expressed as mean and SEM. Comparisons of LDI ratios between time-points were performed by Mann Whitney U test. $P<0.05$ indicated by * and ns indicates not significant.

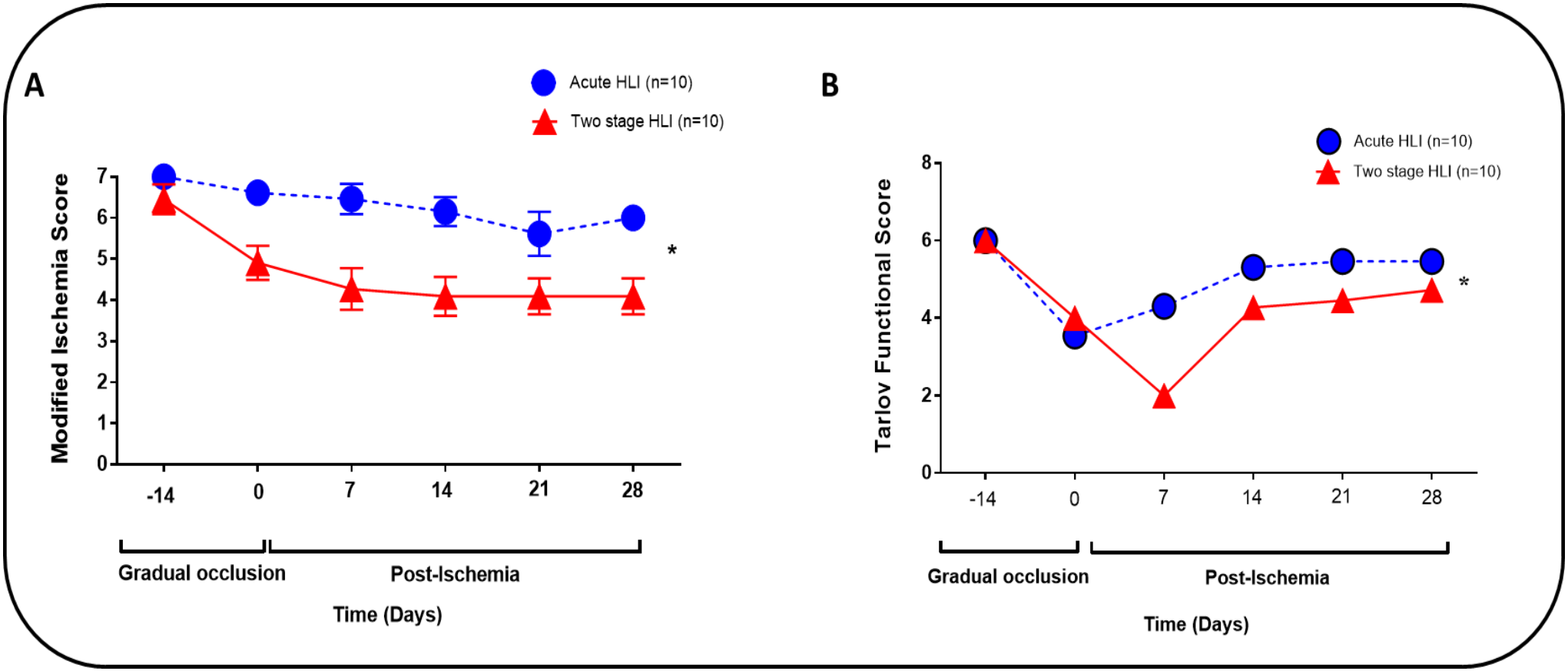


Figure R3.2: Comparison of the severity of limb ischemia and function in the two mouse models using scoring systems. A: Observational assessment of ischaemia in the acute and two-stage HLI models. B: Observational assessment of function in the acute and two-stage HLI models. All data expressed as mean±SEM. Comparisons between groups were performed by 2-way ANOVA and significant difference ($p<0.05$) indicated by *.

3.3.3 Mice with two-stage HLI travelled less distance than mice with acute HLI on the treadmill exercise test

At baseline, similar treadmill distances were travelled by the acute HLI group and the two-stage HLI group (Figure 3.3A). After 4 weeks from ischaemia induction, the two-stage HLI group had significantly greater impairment in treadmill distance travelled than the acute HLI group (*p=0.028*, Figure R3.3A). Comparable treadmill distances were travelled by the acute HLI sham and the two-stage HLI sham groups at baseline and 4 weeks after ischaemia induction (Figure R3.3A).

Mice with acute HLI travelled similar treadmill distance to respective shams on the treadmill test 4 weeks after ischaemia induction (*p=0.818;* Figure R3.3B). The two-stage HLI group travelled less distance in the treadmill exercise test than their respective shams, however the difference was not statistically significant (*p=0.052;* Figure R3.3B)**.** Mice with two-stage HLI travelled significantly less distance on the treadmill test than mice with acute HLI 4 weeks after HLI induction (*p=0.009;* Figure 3.3B).

3.3.4: Mice with two-stage HLI had similar physical activity impairment to mice with acute HLI as assessed by the open field test

Significantly less (*p=0.004)* total distance was travelled in the OF arena by mice with two-stage HLI compared to their respective shams (Figures R3.3C). Mice with two-stage HLI travelled less than the acute HLI group within the arena, however the difference was not statistically significant (*p=0.156*; Figure R3.3C).

3.3.5: Running wheel exercise training had no effect on limb perfusion in mice with two-stage HLI

Limb blood supply in mice with two-stage HLI receiving running wheel exercise training was comparable to control mice with two-stage HLI without access to running wheel exercise throughout the experimental period (*p=0.700*; Figure R3.4**)**.

3.3.6: Running wheel exercise training increased treadmill walking distance in mice with two-stage HLI

Distance travelled on the treadmill exercise performance test was significantly greater in mice with two-stage HLI receiving running wheel exercise training than control mice with two-stage HLI without access to running wheel exercise (*p=0.003*; Figure R3.5).

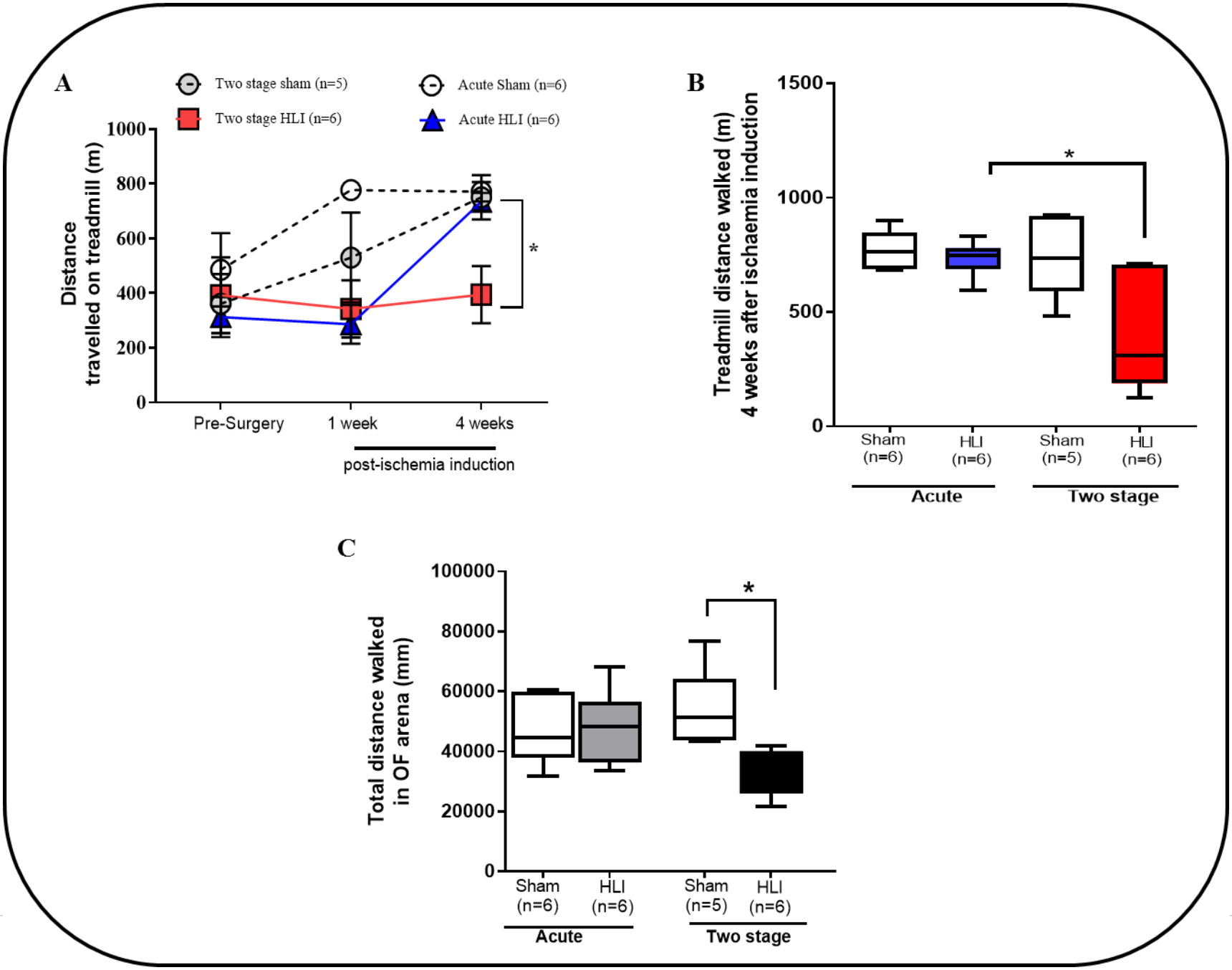

A
Two stage sham (n=5)
Two stage HLI (n=6)
Acute Sham (n=6)
Acute HLI (n=6)
Distance travelled on treadmill (m)
1000
800
600
400
200
0
Pre-Surgery
1 week
4 weeks
post-ischemia induction
*
B
Treadmill distance walked (m) 4 weeks after ischaemia induction
1500
1000
500
0
*
Sham (n=6)
HLI (n=6)
Sham (n=5)
HLI (n=6)
Acute
Two stage
C
Total distance walked in OF arena (mm)
100000
80000
60000
40000
20000
0
*
Sham (n=6)
HLI (n=6)
Sham (n=5)
HLI (n=6)
Acute
Two stage

Figure R3.3. Treadmill and open field assessment of limb function in HLI models. A: Treadmill walking distance in the acute HLI and two-stage HLI groups and their respective sham controls. B: Total distance travelled in the open field test in the acute HLI and two-stage HLI groups and their respective sham controls. Data expressed as mean±SEM. Data compared between groups by two-way ANOVA (A) or Mann Whitney U test (B). *P* value of less than 0.05 considered significant and indicated as ***.**

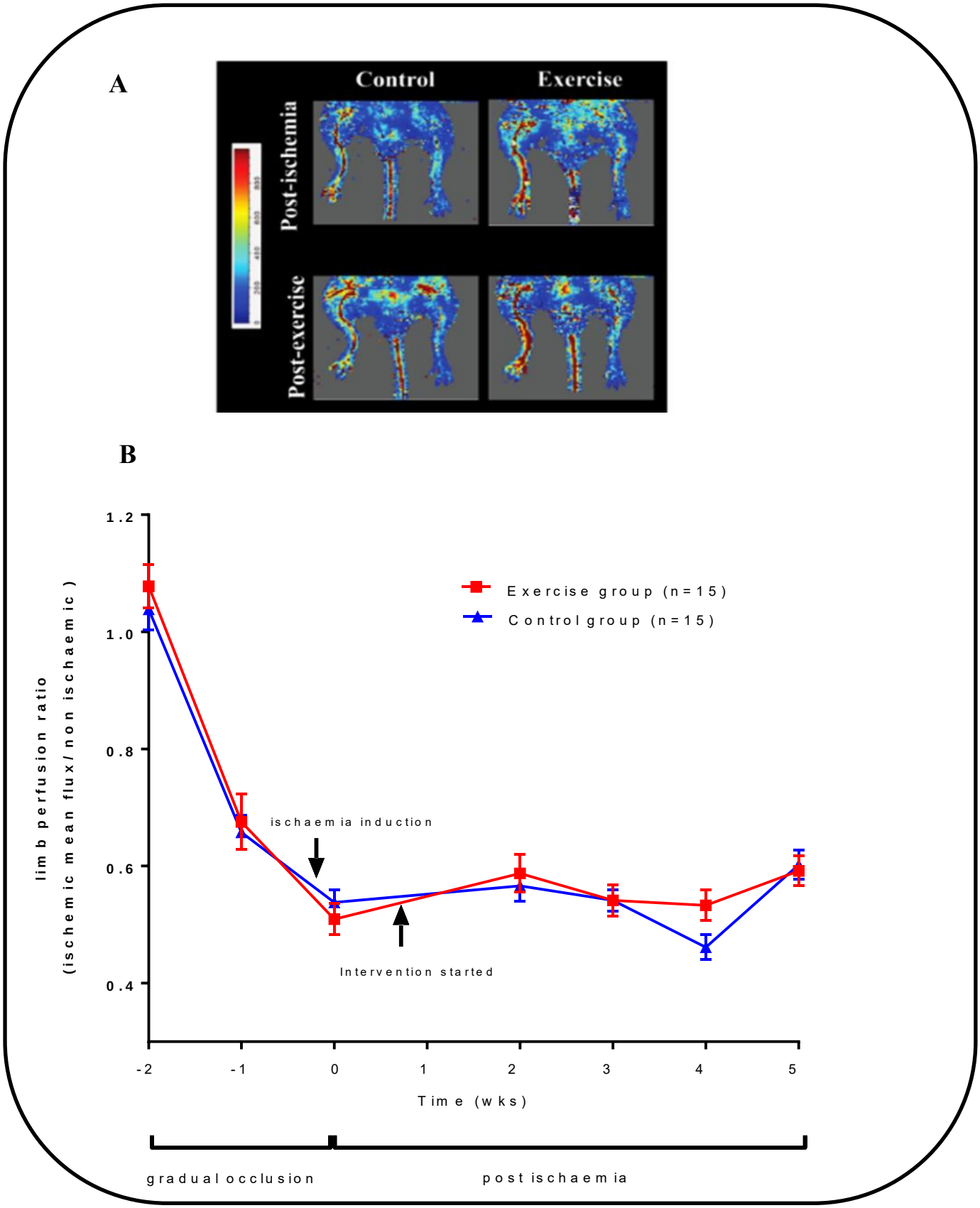


Figure R3.4: The effect of running wheel exercise training on limb blood supply in the two-stage HLI model. A*:* Representative laser Doppler images at ischaemia induction and 4 weeks after exercise intervention. B: Laser Doppler imaging limb perfusion ratios in mice receiving exercise and control. Data expressed as mean ±SEM. LME analysis was performed to compare differences between groups and $p<0.05$ was considered significant.

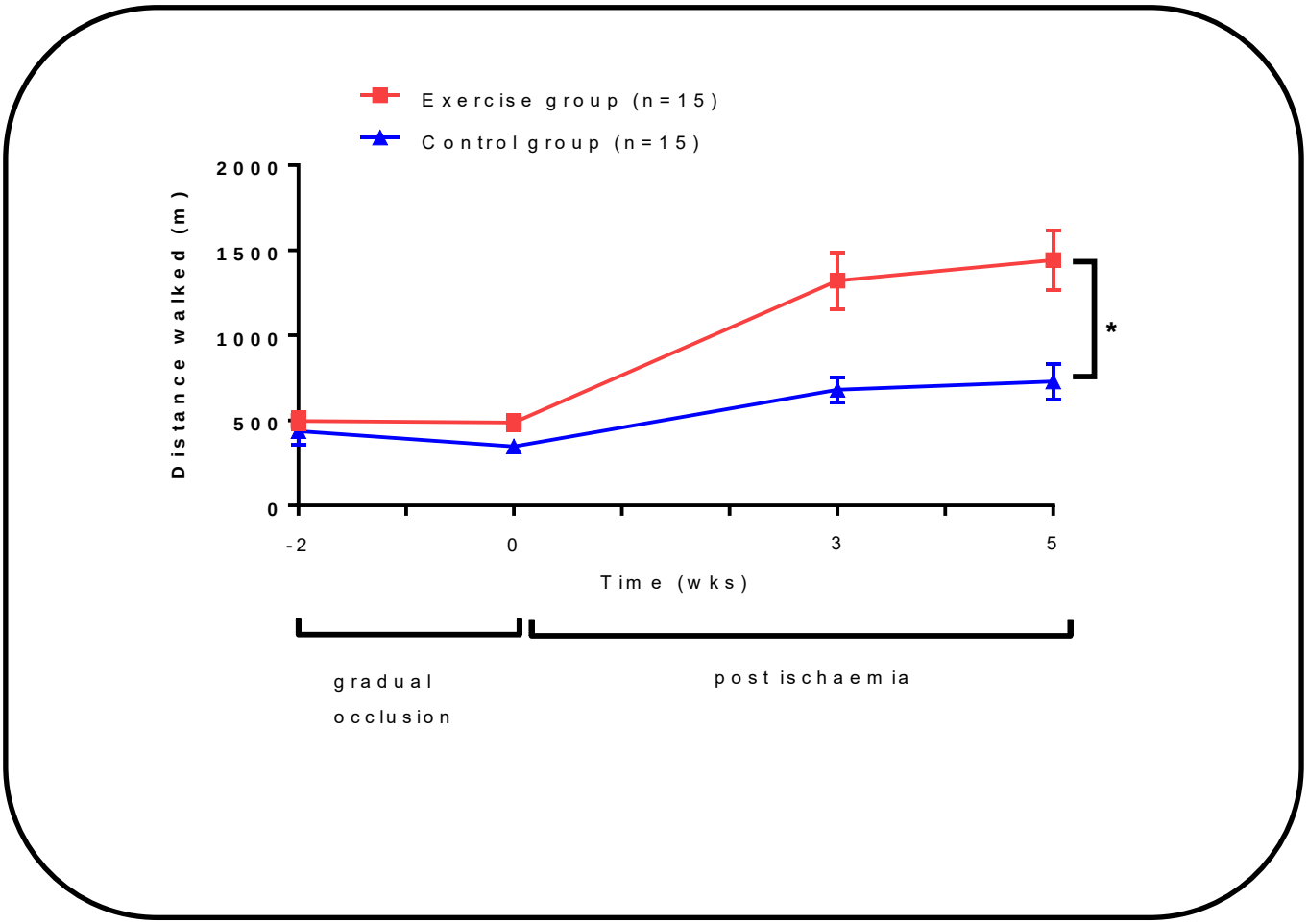


Figure R3.5 The effect of running wheel exercise training on treadmill walking distance in the two-stage model of HLI. Treadmill walking distance in mice receiving exercise intervention and control. Data expressed as mean ±SEM. LME analysis was performed to compare data between groups through the interaction of time and groups over the experimental period, $p<0.05$ was considered significant indicated by * .

3.4 Discussion

The main findings of the studies in this chapter were:

1) The new mouse model of two-stage HLI demonstrated more severe and prolonged ischemia than the acute model of HLI.

2) The two-stage HLI model showed greater ambulatory impairment than the acute HLI model evidenced by the functional scoring assessment and the treadmill test.

3) Exercise intervention after ischaemia induction in the two-stage HLI model promoted treadmill performance independent of a change in limb blood supply.

Previous studies have suggested that ameroid constrictors gradually narrow the FA within 2 weeks resulting in the development of mild ischemia (Tang, Chang et al. 2005, Yang, Tang et al. 2008). Due to the mild degree of ischaemia achieved through ameroids, in the present study a two-stage method was employed combining slow FA constriction with ameroids for 2 weeks followed by excision of the FA. The slow ameroid constriction was used to minimise shear stress which would otherwise result in rapid recovery of blood flow. This was followed by the excision of the FA to mitigate blood supply through collaterals arising from FA in order to achieve severe ongoing ischaemia. In the two-stage model developed in the current study, ameroid constriction alone for 2 weeks did not significantly decrease perfusion compared to blood supply in the hindlimb before surgery. However, the second stage of the FA excision resulted in severe ischaemia to the hind limbs compared to ameroids alone. The results showed that this two-stage method of establishing ischaemia caused ongoing severe ischaemia compared to the currently used FA ligation and excision model. Previous studies report that the ligation and excision of the FA in the acute HLI model leads to increased fluid shear stress within the limb collateral arteries resulting in altered gene expression patterns through shear stress responsive elements which promote arteriogenesis and angiogenesis (Topper and Gimbrone 1999, Garcia-Cardena, Comander et al. 2001, Tang, Chang et al. 2005). Subsequently blood flow is restored rapidly within acute HLI models. Simulating the chronic onset of ischaemia using ameroid constrictors reduces shear stress related promotion of blood flow recovery (Tang, Chang et al. 2005, Yang, Tang et al. 2008). In comparison to the acute model of HLI, the two-stage model of HLI showed minor blood flow recovery immediately following ischaemia induction and after 1 week the severity of HLI persisted. In line with the finding of severe and prolonged ischaemia assessed by LDI in the new mouse model, clinical signs manifested as a result of ischaemia induction was greater in the two-stage HLI model as

assessed by clinical scoring. Ischaemia induction in the two-stage HLI model group resulted in inflammation and toe necrosis which persisted until the experiment endpoint. Together with the main technique of ischaemia induction, the new experimental model which was developed incorporated additional characteristics of PAD patients including atherosclerosis and old age. Atherosclerosis is the leading cause of PAD and atherosclerosis simulating mouse models such as *ApoE*$^{-/-}$ mice and low-density lipoprotein receptor knockout mice have been rarely employed in previous studies in the acute HLI model (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). *ApoE*$^{-/-}$ mice develop significant atherosclerotic lesions spontaneously and exhibit delayed recovery from ischaemia (Kang, Albadawi et al. 2008, Lotfi, Patel et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016).

Greater functional impairment was observed in the two-stage HLI model than the acute HLI model through the clinical scoring method. It is not common for pre-clinical studies to quantitatively assess functional impairment in animal models of HLI and functional assessments mirroring the clinical trial assessments are lacking in pre-clinical studies of HLI. The treadmill test and six-minute walk test are used in clinical trials to assess walking impairment in PAD patients. A few studies have employed treadmill tests for assessing function in mouse models of HLI (Zhong, Eliceiri et al. 2003, Baltgalvis, White et al. 2014, Marcinko, Bujak et al. 2015, Deng, Yang et al. 2016, Krishna, Omer et al. 2016). In the current study assessment of treadmill performance demonstrated the two-stage model of HLI had greater treadmill walking impairment than the acute model of HLI. This suggests that the treadmill test is a potential method of assessment for functional impairment and could be used to test interventions aimed at improving function in mouse models of HLI. The OF has been suggested to mirror the six-minute walk test in clinical trials. Exploring functional impairment in the two-stage HLI model with the open field test did not identify significant impairment in the two-stage HLI model compared to the acute HLI model. The six-minute walk test in patients is influenced by musculoskeletal, volitional factors and cardiovascular capacity (Lancaster 2018). In animal models of neuromuscular disease, the OF test is widely used for assessing locomotive impairment (Ijomone, Olaibi et al. 2014) (Tatem, Quinn et al. 2014). The test is behavioural and can be quite variable as it is influenced by a multitude of external factors. For example, this behaviour can be influenced by exploratory drive, anxiety, circadian rhythm, environmental factors, genetic background, in addition to motor output (Prut and Belzung 2003). As a result, it can be difficult to distinguish if changes in locomotive or behavioural activity levels are related to changes in muscle function (Tatem, Quinn et al. 2014). The finding

in the current study does not support the use of the OF test to distinguish functional impairment in mouse models of HLI.

The demonstration of functional impairment and similar responses to known interventions in humans are important characteristics of an ideal mouse model for PAD (Krishna, Omer et al. 2016). Patients with PAD have markedly reduced health-related quality of life largely related to impaired walking ability (Hamburg and Balady 2011, Brostow, Petrik et al. 2017). A considerable body of evidence supports the clinical benefits of a supervised exercise program in improving pain free walking performance, physical capacity and quality of life (Brass 2013, Hiatt, Armstrong et al. 2015). The Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease (TASC II) have declared that the evidence supporting supervised exercise therapy in the treatment of claudication is sufficiently robust to merit a Level I recommendation (Norgren, Hiatt et al. , Brass 2013). The mechanisms associated with improvements in limb symptoms as a result of an exercise intervention are not clearly understood. The majority of studies in patients have shown little or no increase in blood flow assessed by ABPI following an exercise programme, even when significant improvements in walking ability have been reported (Stewart , Hiatt et al. 2002). Studies have consistently shown a poor correlation between leg blood flow assessed by ABPI and walking ability (Parmenter, Raymond et al. 2010). As there is currently little evidence to support an increase in blood flow as a major factor in the increase in walking ability after training, other mechanisms may account for the improvements seen (Hiatt, Armstrong et al. 2015). In experimental models of acute HLI, exercise training augments blood supply to ischaemic muscles (Yang, Dinn et al. 1990, Yang, Prior et al. 2008, Cheng, Kuzuya et al. 2010, Rokutanda, Izumiya et al. 2011). In acute HLI, blood flow recovery to exercise in ischaemic tissues involves multiple complex processes. Angiogenesis and arteriogenesis are stimulated in ischaemic tissues in the acute HLI model by upregulated growth factor activity and increased NO bioavailability stimulating gains in collateral blood flow (Hamburg and Balady 2011). In the present study, exercise training in the two-stage HLI model improved treadmill walking distance without improving limb blood supply. This is a finding similar to that described in PAD patients (Watson, Ellis et al. 2008, Parmenter, Raymond et al. 2010, Malgor, Alahdab et al. 2015). Therefore, validates the two-stage HLI model for assessing interventions for improving functional impairment. Exercise intervention in this study commenced 5 days after ischaemia induction. One of the major limitations of pre-clinical HLI studies currently is study design in which the administration of the interventions is usually started prior to HLI induction

(Takahashi, Shibata et al. 2015, Mohamed Omer, Krishna et al. 2016). This prophylactic approach does not reflect the intervention in patients who seek treatment after established ischaemia. To reflect this, in the current study exercise training was started after established ischaemia which was seen to be prolonged over 5 weeks after induction.

Strengths of the studies reported in this chapter include the establishment of a new mouse model through two-stage induction of ischaemia with severe prolonged ischaemia and functional impairment; testing mice prone to atherosclerosis and mice with advanced age in the newly developed mouse model; the use of a clinically relevant treadmill test to assess functional impairment, the characterisation of the effect exercise training in the newly developed model by commencing exercise training after achieving ischaemia; and the finding that exercise promotes function unrelated to improvement in blood supply similar to patients' response.

The current study utilised older mice to better simulate the characteristics of PAD patients. A limitation in this study was that 12 month old mice were used in the development of the 2 stage model but 6 month old mice were used in examining the effect of an exercise intervention. However, all mice used were relatively older than typically used in experimental work . Future work examining different ages of mice could determine the effect of age on ischaemia in the two stage mouse model of HLI. Although this study used *ApoE*$^{-/-}$ mice prone to atherosclerosis a comparison was lacking with non-atherosclerotic C57.Bl6 to determine blood flow recovery in the two stage model of HLI. A future study examining this is suggested. The new two stage model attempts to mimic the slow onset of ischaemic in patients, however the ameroids occlude the artery within 2 weeks. In patients it may take numerous years for atherosclerotic occlusions to occlude the arteries resulting in symptoms. Therefore, new methods to mimic chronic occlusions need to be explored in future studies. This study also did not compare the new two stage model to a single stage ameroid model over the experimental period.

It is also known, that other factors including diabetes and smoking affect blood flow recovery to limb ischaemia in patients, although an exhaustive examination of all of these risk factors was beyond the scope of the current study. In addition, it is known that angiogenic capability of some other mouse strains (e.g. BalbC) is lower than that of C57.Bl6 employed here. It is possible that the surgical procedures proposed in this study may be more effective when combining multiple co-morbidities in other strains, and future research investigating this is

warranted. Furthermore, future studies are needed to characterise the underlying cellular and molecular mechanisms related to severe prolonged ischaemia in the two-stage model and the characterisation of the cellular and molecular mechanisms leading to improved treadmill walking with exercise in the two-stage HLI model.

Current animal models to assess treatments for PAD have many limitations (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Although a single approach in animals cannot fully recapitulate the human condition, an experimental model which can recapitulate the salient features of the disease will aid in the relevant interpretation and translation to clinical insights of potential treatments. In this study, a new mouse model of slow onset severe HLI was developed.

In conclusion, the two-stage HLI model achieves similar degree of hind limb ischaemia as the one stage model. The two stage model showed severe and prolonged ischaemia and ambulatory impairments compared to the one stage model of recovery from ischaemia and ambulatory impairment. Furthermore, the new two-stage HLI mouse model showed a clinically relevant response to exercise training in that exercise training improved treadmill walking distance independent to a change in limb blood supply assessed by LDI.

Chapter 4
Characterisation of the effect of metformin on established experimental limb ischaemia

4.1 Introduction

There is currently a lack of effective drugs for leg ischemia and no effective drug development pipeline (Gerhard-Herman, Gornik et al. 2016). Pharmacological interventions that have been successful in the acute HLI model have not proved to be effective in large clinical trials (Annex 2013, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Moxon et al. 2015, Mohamed Omer, Krishna et al. 2016). The inability to translate findings from previous pre-clinical studies to patients could be due to the lack of relevant experimental limb ischaemia models and appropriate study design. Current HLI models do not simulate a more typical patient presentation of PAD, such as an ongoing state of ischemia arising from chronic ischaemic insult to the limb, old age and atherosclerosis (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). In contrast to human PAD, most previous pre-clinical trials are designed to assess potential interventions within model rodents in which ischaemia is not established (Takahashi, Shibata et al. 2015). This prophylactic approach does not represent the clinical situation where treatment usually occurs following the presentation and diagnosis of established chronic ischemia. In addition, many previous studies are not designed to include appropriate measures to limit potential observer bias such as blinding the investigators who are conducting outcome assessments, and lack of allocation randomisation (Mohamed Omer, Krishna et al. 2016).

In Chapter 3 of this thesis, a new mouse model of persistent limb ischaemia was established and validated in order to better assess interventions. Repurposing drugs which may have angiogenic potential could provide a rapid approach for implementing successful interventions to manage limb ischaemia due to the benefits of approval status, established dose and safety profile and availability. The biguanide metformin is used to manage hyperglycaemia in type 2 diabetes and has recently gained significant attention for being repurposed for several metabolic diseases (NIH 2018).

Small uncontrolled clinical studies have suggested that metformin can improve blood supply in patients with PAD (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992). Suggested mechanisms for the proposed beneficial effects of metformin include promoting arteriogenesis, stimulating angiogenesis, improving microcirculatory function, limiting oxidative stress and favouring mitochondrial biogenesis (Hamburg and Balady 2011, Steven, Daiber et al. 2017). Metformin has been recently reported to improve hind limb blood supply within the acute HLI model by increasing AMPKα and e-NOS activity within ischaemic

muscles in order to promote angiogenesis (Takahashi, Shibata et al. 2015). Further data also suggest that metformin may attenuate oxidative stress by, for example, downregulating expression of thioredoxin interacting protein (TXNIP) which has been suggested to be a critical protein in promoting severe limb ischemia in diabetes (Chong, Chan et al. 2014, Zhang, Pang et al. 2015). Oxidative stress and mitochondrial biogenesis have been previously shown to be associated with muscle changes relevant for functional improvements resulting from limb ischaemia.

4.1.1 Hypothesis and aims of study

The specific hypothesis and aims of this Chapter were:

4.1.1a Hypothesis

Primary: Metformin would improve blood supply in the two-stage mouse model of limb ischaemia

Secondary

1) AMPKα activity and e-NOS activity would be increased in mice receiving metformin

2) Oxidative stress marker TXNIP would be decreased and mitochondrial biogenesis marker PGC1α would be upregulated in mice receiving metformin

Tertiary: The improvement in blood supply caused by metformin would improve treadmill walking distance after established ischaemia

4.1.1b Aims

The aims of this chapter were:

Primary: To examine the effect of metformin administration on blood supply in the experimental model of ongoing limb ischaemia.

Secondary: To assess the effect of metformin on AMPKα activity, e-NOS activity and the differential protein and gene expressions of TXNIP and PGC1α in the experimental model of ongoing limb ischaemia.

Tertiary: To examine the effect of metformin administration on treadmill exercise performance in the experimental model of ongoing limb ischaemia.

4.2 Methods

4.2.0 Ethics and mouse husbandry

Institutional ethics was obtained for the studies presented in this Chapter (Appendix) and mice were maintained as described in Chapter 2.1.

4.2.1 Study design, group allocation and protocol

This was a placebo-controlled trial utilising the two-stage mouse model. Unilateral left HLI was induced in 31 male *ApoE*$^{-/-}$ mice (n=31) through the two-stage HLI induction method. Five days after HLI induction, mice commenced receiving metformin (n=16; 300mg/kg/day) or vehicle control (n=15; distilled water). The dose of metformin was chosen since it is estimated to equate to a daily dose of approximately 1500mg in humans which is the most common dose used (Christiansen, Ehrenstein et al. 2015). The mouse equivalent dose was estimated by normalisation to the body surface area using the United States Food and Drug Administration recommended conversion factor of 12.3 (Rockville 2005). Previous studies have suggested that using this conversion factor, murine plasma levels of metformin are achieved which correlate well with ranges achieved within human plasma (Foretz, Guigas et al. 2014). Drug or vehicle control administration by oral gavage was commenced 5 days after completing ischemia induction (Figure M4.1), since a preliminary experiment demonstrated that the severity of HLI was stable at this stage. An independent research assistant (Ms Anne Kraueter) performed blinded administration of metformin or vehicle but played no further part in the study. Two separate vials of metformin or vehicle was freshly prepared by the main investigator before each administration and labelled in a way the person administering did not know what was administered or how many interventions were tested. Drug administration was continued for 4 weeks and then mice were euthanised by CO_2 asphyxiation and lower limb muscle tissue samples were harvested in OCT for Western blot assays or RNA later for qPCR. OCT

embedded samples were snap frozen in liquid nitrogen and stored in -80°C and RNA later samples were stored at -20°C for later analysis.

The primary outcome measure was blood supply measured by LDI. During the assessment and analysis of LDI, the group allocation was blinded to the investigator. Secondary outcome measures were treadmill exercise performance test, protein expression analysis of AMPKα. phospho-AMPKα, e-NOS, phospho-eNOS, TXNIP and PGC1α by Western blotting; NO assay, and blood glucose assay and gene expression assays of AMPKα, e-NOS, TXNIP and PGC1α by qRT-PCR.–Similar to the primary outcome of LDI, treadmill exercise performance was performed with the group allocation blinded to the investigator.

4.2.2 Sample sizes

Sample size for this study was estimated for the primary aim using the outcome measure of LDI. For calculating the expected outcome for the metformin administered group and vehicle control group, the LDI results of a previous group of mice in which the acute HLI model was employed were used (Takahashi, Shibata et al. 2015). In that study, 10 mice were imaged for 28 days after ischemia induction and mean (SD) flux ratio of the control group was 0.51 (0.20). It was assumed that in the metformin administered mice, ischaemic hind limb blood supply would be increased by 30% which is suggested to improve ischaemic limb impairment and tissue injury (Takahashi, Shibata et al. 2015). Based on these assumptions and aiming to achieve 80% power with an alpha of 0.05, it was estimated that 12 mice were required in each group (estimated with G* power). Sample sizes were adjusted by adding approximately 30% to this estimate, in order to account for exclusions of animals during the experiment due to technical difficulties or unexpected mortality or morbidity which might be experienced.

4.2.3 HLI induction

Unilateral left HLI was induced in all experimental mice (n=31) through the two-stage HLI induction method as described in chapter 2.

4.2.4 Laser Doppler imaging

LDI was performed as described in chapter 2. LDI measurements were carried out at baseline, 7 days after ameroid placement, after ischaemia induction and weekly after starting metformin

or vehicle administration for 4 weeks (Figure M4.1). The groups were blinded during assessment and analysis.

4.2.5 Functional assessment with a treadmill test

The treadmill test was performed with mice as described in chapter 2. Treadmill testing was carried out at baseline (before first surgery), 2 days after ischaemia induction and, 3 and 5 weeks after commencing metformin or vehicle administration. The groups were blinded during assessment and analysis.

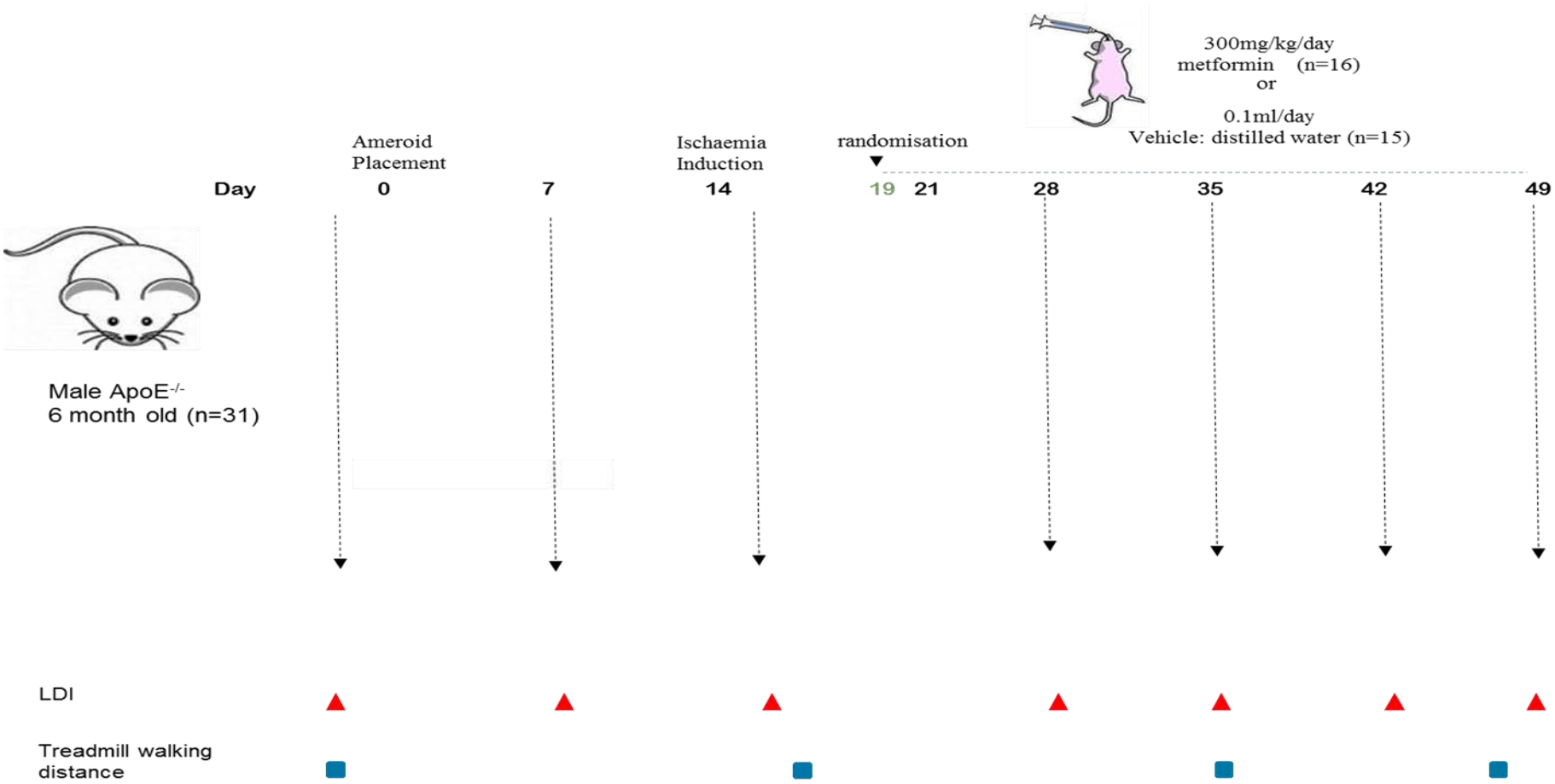


Figure M4.1: Study design for examining the effect of metformin on HLI. Mice were randomised 5 days after ischaemia induction and started on daily intervention with metformin or vehicle. LDI assessments were carried out at baseline, days 7, 16, 28, 35, 42 and 49. Treadmill walking distance tests were performed at baseline, days 16, 35, and 45.

4.2.6 Western blotting

Western blotting was performed to examine the protein expression of AMPKα. phospho-AMPKα, e-NOS, phospho-eNOS, TXNIP and PGC1α in mice receiving metformin and vehicle control. Antibodies and dilutions used are shown in Appendix (Table A1).

4.2.6a Sample preparation

The ischaemic gastrocnemius muscles which were harvested at euthanasiaand stored in OCT compound (ProSciTech) at -80^{0}C were used for Western blotting. Each tissue sample was thawed on ice, rinsed in PBS, transferred to a clean microfuge tube and snap frozen in liquid nitrogen. The tissue was then minced manually with a pestle, 500µl of ice-cold radio-immunoprecipitation assay (RIPA) buffer (Cell Signalling Technology) containing protease inhibitors (Roche) and phosphatase inhibitors (PhoSTOP) was added to the minced tissue and homogenised with the pestle attached to a handheld drill. The homogenised samples were centrifuged for 5 minutes at 80000xg at 4°C. The supernatant was collected and protein concentration was quantitated by the BioRad protein assay (BioRad, USA).

4.2.6b Protein estimation by BioRad protein assay

IgG standards (range 0-8µg) were prepared by diluting IgG (1mg/ml) with milli-Q water to construct a standard curve. A small volume of each sample (2µl) was diluted to 1:1000 in milli-Q water and used for the assay to determine the protein concentration. Each sample (160µl) or standard (160µl) was mixed with 40µl Bradford reagent in a flat bottom 96-well plate. The solutions were mixed, followed by 30 minutes of incubation in the dark. The optical density (OD) of the wells were read at 595nm in the Omega plate reader. GraphPad prism software was used to interpolate unknown concentrations of the diluted samples using the standard curve. The interpolated values were multiplied by the dilution factor (x1000) for the protein concentrations of the extracted proteins of the samples.

4.2.6c Electrophoresis separation of proteins

Protein samples (20µg) were mixed with Laemmli buffer containing dithiothreitol (DTT; 0.39mg per 1ml of Laemmli buffer; Bio-Rad, USA) and boiled at 95°C for 5 minutes. Samples (15µl) were loaded into the wells of 4-15% SDS-polyacrylamide electrophoresis pre-cast gels (BioRad), along with Precision Plus Protein™ WesternC™ Protein Standard (BioRad). The

gel was run in 1xTris/glycine/SDS electrophoresis buffer (BioRad) at 80V for 10 (until the proteins begin to enter the resolving gel) minutes and until fully resolved (until the dye front disappeared) at 100V.

4.2.6d Transfer of proteins from gel to membrane

The wet transfer technique was employed to tranfer the proteins from gel to membrane. Following the separation of the proteins, the gel was placed in transfer buffer containing 10% methanol. Polyvinylidene fluoride (PVDF) (Biorad) or PVDF FL (Immobilin FL, Licor) membranes were activated in 100% methanol, and filter paper and fibres pads were moistened in transfer buffer. The transfer sandwich was assembled as shown in figure M4.2 with the blot on the cathode and the gel on the anode and ensured no air bubbles are trapped in the sandwich. The cassette was placed in the transfer tank containing Transfer buffer, an ice block was placed in the tank and the transfer was carried out at 100V for 60 minutes. After transfer, PVDF membranes were Ponceau stained to confirm successful transfer. Membranes were cut between bands to separate the different regions of membrane of the protein to be investigated according to molecular size using the markers. PVDF FL membranes were stained with Revert protein stain (Licor) and visualised for total protein by the Odyssey imaging system.

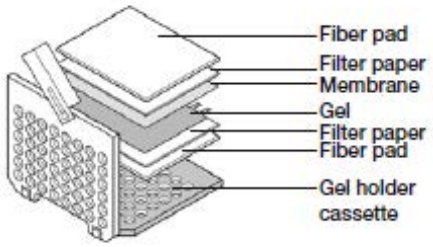


Figure M4.2 Western blot wet transfer sandwich assembly (Bio-Rad 2016)

4.2.6e Protein detection and visualisation: chemiluminescent method

PVDF membranes were recharged in 100% methanol and placed in TBS-T (0.1% tween) in a low speed shaker. Non-specific sites were blocked with 5% ECL prime blocking agent for 60 min at room temperature. The blots were then incubated with the primary antibody overnight at 4°C on a low speed shaker. Membranes were washed 3 times, 10 minutes each time in TBS-T and incubated with the HRP conjugated secondary antibody (goat anti-rabbit; 1:1000) (DakoCytomation, Denmark) in room temperature on the low speed shaker for 2 hours. This

was followed by 3 washes with TBS-T for 10 minutes each. After which the membrane was placed in TBS. The membrane was imaged using a LiCor Odyssey scanner and band intensities were quantified using the Odyssey software.

4.2.6f Protein detection and visualisation: fluorescent method

PVDF FL membranes were recharged in methanol and incubated with Odyssey blocking buffer (Li-Cor)for 1 hour prior to incubation with rabbit polyclonal antibodies directed against TXNIP (anti mouse TXNIP) and PGC1α (anti-mouse PGC1α) overnight at 4°C. After washing, blots were incubated with donkey anti-rabbit secondary antibodies conjugated with IRDye 800CW (Licor) for 1 hour in room temperature. Membranes were washed in TBS-T and imaged using a LiCor Odyssey scanner and analysed using the Odyssey software.

4.2.7 Plasma nitric oxide assay

At euthanasia, blood was collected by cardiac puncture into heparin coated tubes (BD Microtainer). Platelet poor plasma was separated by centrifugation of blood at 4000 × g at 4^0C for 10 minutes followed by a further 10 minutes centrifugation at 10,000 × g at 4^0C. NO levels in plasma was determined indirectly by measuring the concentration of the stable end products nitrate and nitrite using a commercial kit (Cayman) based on the Griess reaction. The protocol provided by the manufacturer was followed. Nitrate standards (range 0-35 µl) were prepared by diluting 200 µM reconstituted nitrate standards with assay buffer to construct a standard curve. Samples or standards (40 µl) were assayed in triplicate in a flat bottom 96 well plate (Cayman) and each was mixed with 40 µl assay buffer solution. Enzyme co-factor mixture (10 µl) and nitrate reductase mixture (10 µl) were subsequently added to the wells. The plate was then incubated at room temperature for 3 hours in the dark. After incubation, Griess reagent R1 (50 µl) was added to each of the wells followed immediately with Griess reagent R2 (50 µl) and colour was allowed to develop for 10 minutes at room temperature. The absorbance was measured at 540 nm using an Omega plate reader.

4.2.8 Plasma glucose measurement

Glucose measurements were performed on plasma samples prepared at euthanasia. Samples were vortexed and glucose concentrations were measured with an Accu-Chek Performa glucometer and test strips. Briefly, 1µl of sample was placed on a fresh test strip, inserted into

the glucometer and displayed reading was recorded. Each sample was tested with a new test strip.

4.2.9 Quantitative real-time polymerase chain reaction assays

Qiagen QuantiTect Primer Assays (Qiagen) were used to assess AMPKα1, PGC1α, TXNIP, e-NOS and GAPDH gene expression (Appendix Table A5).

4.2.9a RNA extraction

Total RNA was isolated from ischaemic gastrocnemius muscles using a RNeasy Mini kit (Qiagen) according to manufacturer's instructions. The tissue was cut into 5mmx5mm piece in a petri dish and placed in an Eppendorf tube. The sample was homogenised in 500µl Qiazol reagent using a sterile pestle and drill and an additional 500µl of Qiazol reagent was added. The samples were left in for 5 minutes at room temperature and 200µl of chloroform (Sigma-Aldrich) was added. The samples were shaken for 20 seconds, left for 3 minutes in a shaker at room temperature. The samples were centrifuged for 15 minutes at 10,000xg at 4°C treating the samples with care not to disturb the phase. The top aqueous phase was removed and the sample was treated with 500 µl of 2-isopropanol for 10 minutes at room temperature. RNA was purified with spin columns in the RNeasy kit (Qiagen, Germany). Briefly, an equal volume of 70% ethanol was added, mixed and transferred to spin columns, then centrifuged at 10,000g for 30 seconds and washed with RW1 buffer twice. The column was washed again with 100% ethanol and the DNAse digestion was carried out for 30 minutes at 37°C, the columns were washed again with RW1 and RPE buffer. The RNA was eluted into ultra clean RNA tubes, in 20µl of RNAse free water. The RNA was quantified using the Nanodrop spectrophometer (Thermoscientific, Australia) at 260/280 nm to assess purity. RNA with an OD of 1.8-2.0 at 260/280 were used for qRT-PCR experiments.

4.2.9b Reaction preparation

The qRT-PCR reactions were performed using QuantiTect SYBR Green one-step RT-PCR assay (Qiagen). Each reaction was performed with 10ng of RNA set up in a reaction volume of 10 µL containing 5 µL SYBR Premix, 0.5 µL primer mix and 0.1 µL reverse transcriptase.

4.2.9c q-RT-PCR cycling conditions

A three step melt thermal cycling program was used: 50°C for 30 minutes for cDNA synthesis, 95°C for 15 minutes (enzyme activation), 40 cycles of 94°C, 15s (denaturation), 55°C for 30s

(annealing), 72°C for 30s (extension). For each gene of interest, the relative expression in each sample was determined by using the concentration-Ct-standard curve method and normalised to the expression of GAPDH. All samples were tested in duplicate.

4.2.10 Statistical analysis

Graphpad Prism V6.0 (GraphPad Software, San Diego, CA) and R studio software programs were used to analyse data. Graphpad was used to construct graphs. Data were tested for normality using D'Agostino-Pearson normality test. Data with normal distribution were expressed as mean ± standard error of mean (SEM) and analysed using parametric tests. Non-normally distributed data were expressed as median and interquartile ranges (IQR) and analysed using non-parametric tests. LME model analysis was used to compare LDI data between groups. For LME analyses variation between individual mice were treated as random effects. LDI data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Interaction between time and treatments were assessed by LME. LME model analysis was used to compare treadmill data between groups. For LME analyses variation between individual mice were treated as random effects. Treadmill data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Data were square root transformed to fit model assumptions. Interaction between time and treatments were assessed by LME. Western blot data, NO assay data, glucose concentration data and qRT-PCR data were compared between groups using a Mann-Whitney U test and expressed as median and IQR. In all cases a p value of <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Metformin administration improved limb blood supply

Blood supply to the hind limbs at baseline was similar between experimental groups before surgery (Figure R4.1). Mice in both groups experienced a significant reduction in hind limb blood supply following ameroid placement and ischaemia induction, and exhibited a comparable level of HLI at the commencement of metformin or vehicle administration (Figure R4.1). Subsequently mice receiving metformin exhibited improved limb blood supply compared to the vehicle control group ($p<0.001$; Figure R4.1. B). Four weeks after receiving interventions, mice receiving metformin had 16.17% greater blood supply to the ischaemic limbs compared to control mice receiving vehicle.

4.3.2 Metformin administration did not significantly affect treadmill performance

Mice receiving metformin and mice receiving vehicle control had comparable distances travelled on the treadmill exercise test throughout the experimental period (Figure R4.2, $p=0.241$).

4.3.3 Phospho-AMPKα/total AMPKα and phospho-eNOS/e-NOS were increased in mice receiving metformin

In mice receiving metformin, AMPKα protein expression levels were similar to controls ($p=0.818$, figure R4.3A*i*). Mice receiving metformin had higher levels of phospho-AMPKα compared to vehicle administered mice ($p=0.041$, Figure R4.3A*ii*). The phospho-AMPKα level relative to total AMPKα expression was significantly greater in mice receiving metformin compared to controls ($p=0.009$, Figure R4.3A*iii*). Higher levels of phospho-eNOS expression relative to total e-NOS expression was observed in mice receiving metformin (p=0.031, Figure R4.3B*iii*). Total e-NOS expression within the ischaemic gastrocnemius muscles was similar in mice receiving metformin and vehicle ($p=0.821$, Figure R4.3B*i*). However, mice receiving metformin had significantly higher levels of phospho-eNOS ($p=0.047$, Figure R4.3B*ii*) than controls. The mRNA levels of AMPKα and NOS3 were similar in mice receiving metformin and vehicle ($p=0.378$, Figure R4.6A; $p=0.442$, Figure R4.6B).

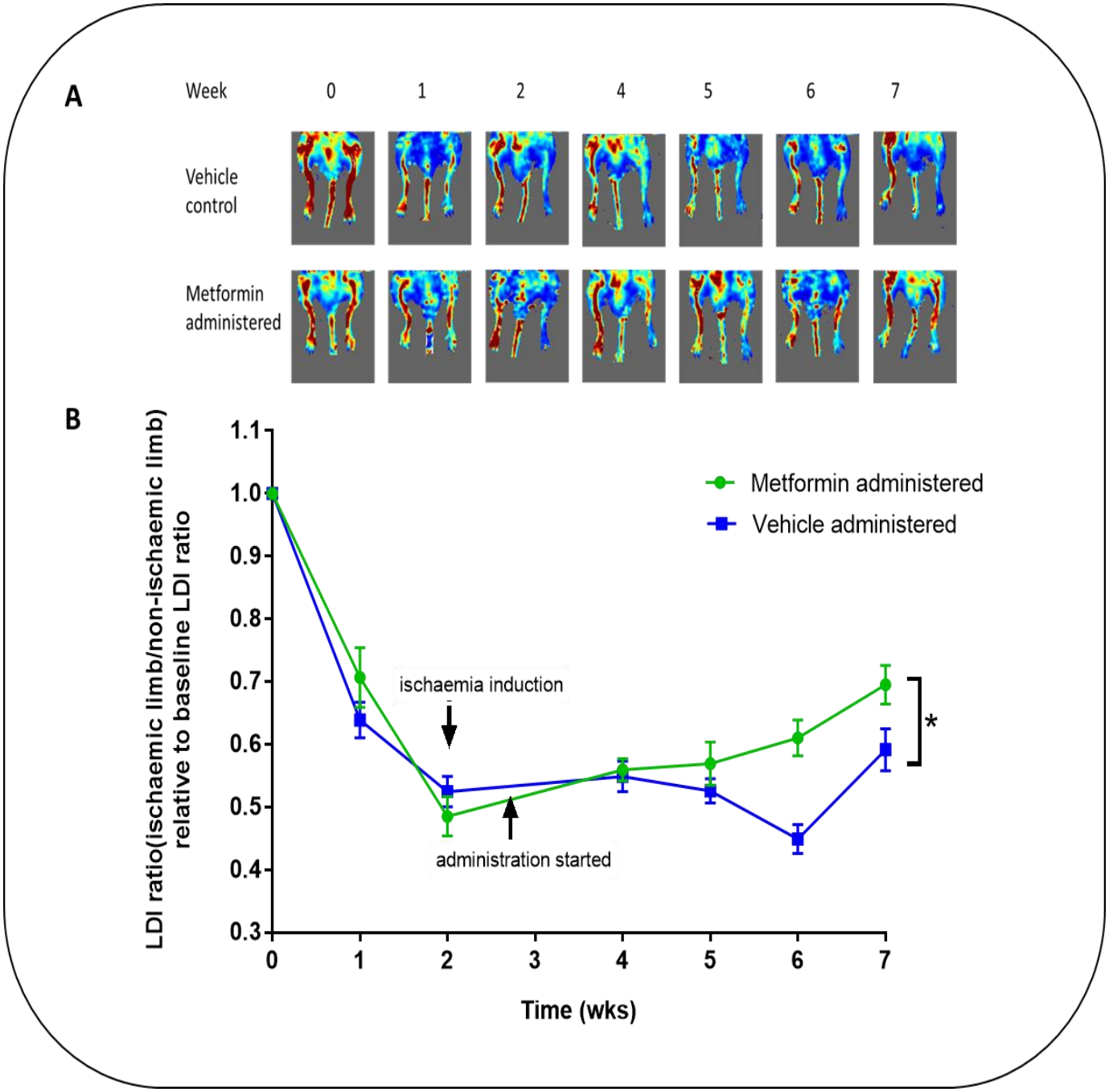


Figure R4.1. Effect of metformin administration on hindlimb blood supply in the two-stage mouse model of PAD. A: Representative laser Doppler images at the assessment times of the intervention groups. B: Laser Doppler imaging limb perfusion ratios in mice receiving metformin (n=15) and vehicle control (n=16). Values are normalised to the means of the baseline for each group and data is expressed as mean ±SEM. LME analysis suggested a significant difference ($p<0.001$) between groups over the experimental period.

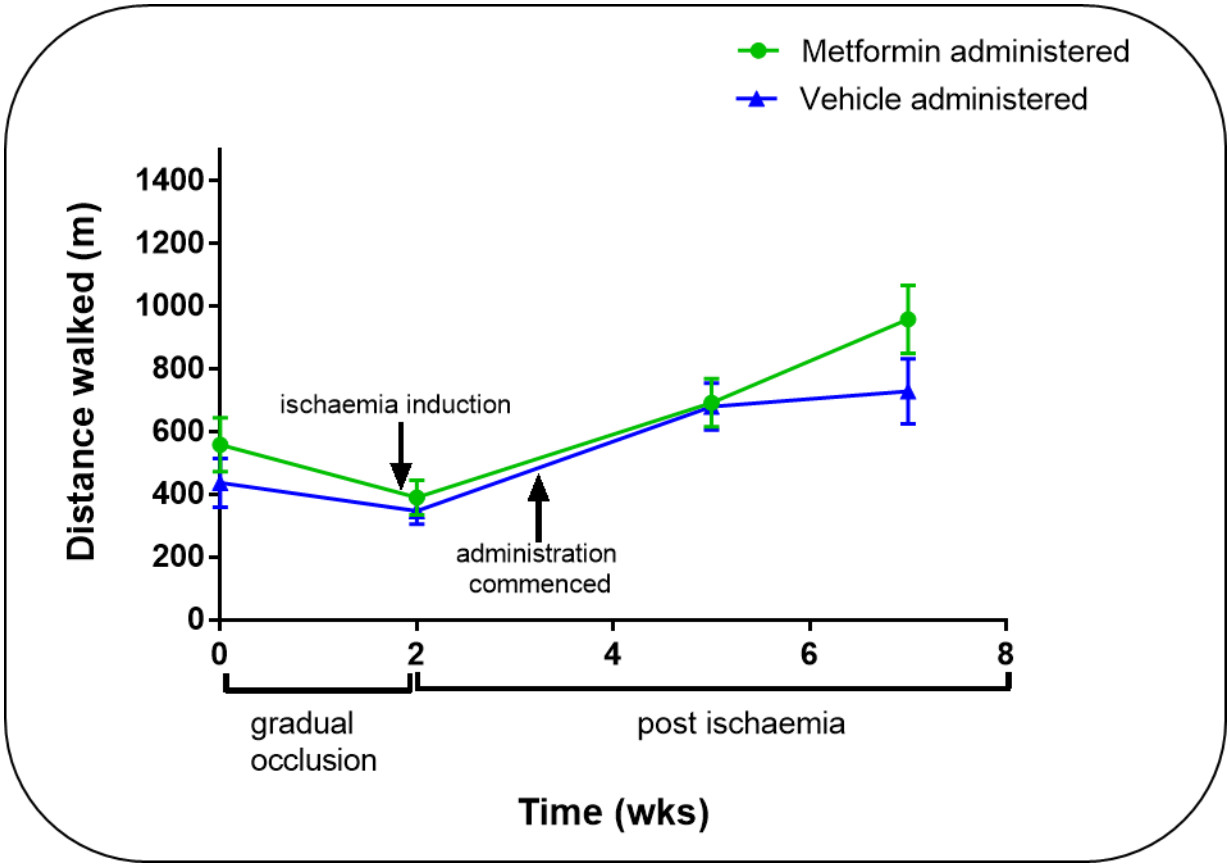


Figure R4.2. The effect of metformin on treadmill exercise performance in the two-stage mouse model of PAD. Treadmill distance travelled by metformin administered (n=15) and vehicle administered groups (n=16). Administration commenced 5 days post ischaemia. Data expressed as mean ±SEM. Data were analysed by LME and square root transformed to fit model assumptions. The treadmill distance travelled showed no significant difference over time (*p=0.241*) in mice receiving metformin and vehicle.

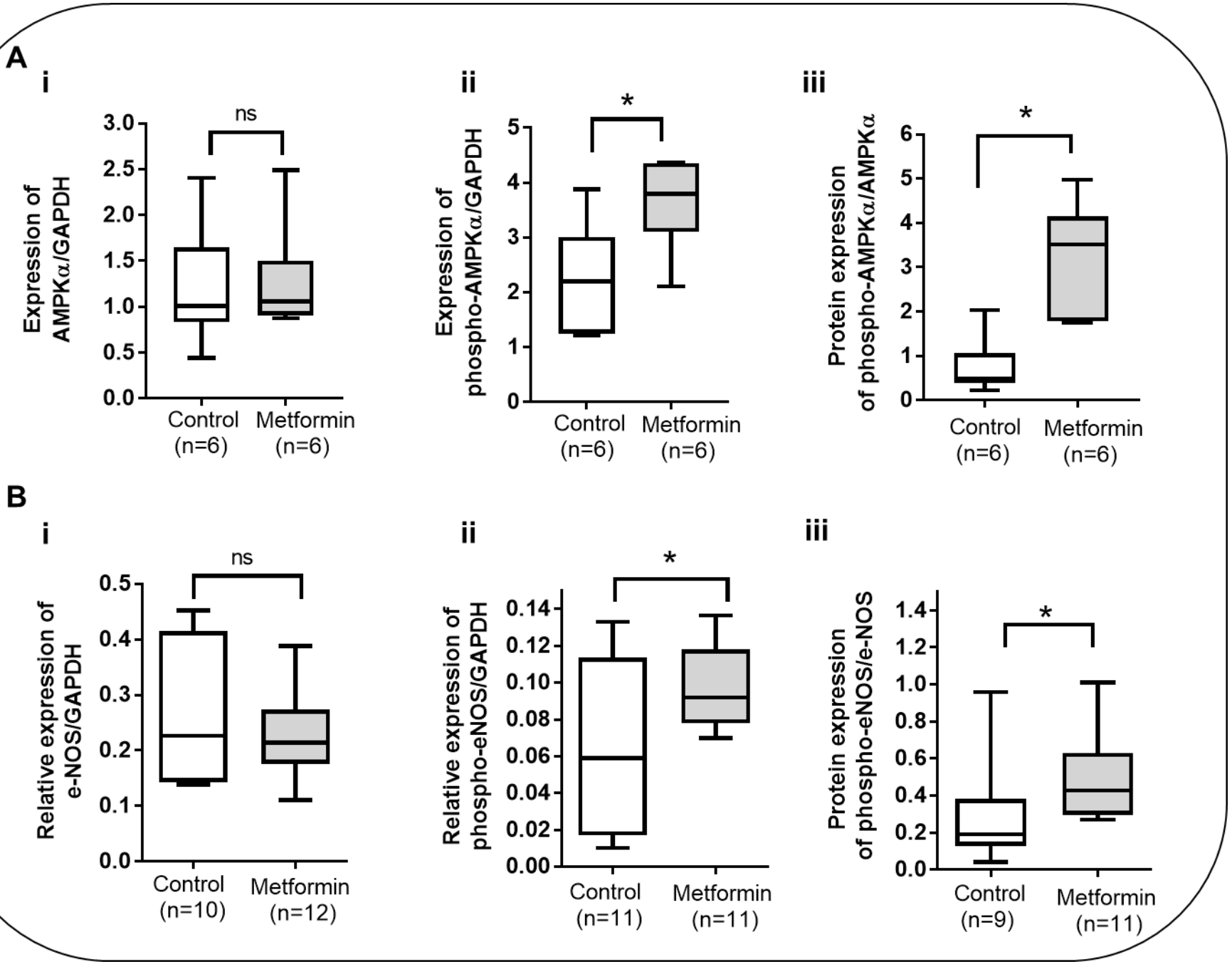

A
i
ns
Expression of AMPKα/GAPDH
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control (n=6)
Metformin (n=6)
ii
*
Expression of phospho-AMPKα/GAPDH
5
4
3
2
1
0
Control (n=6)
Metformin (n=6)
iii
*
Protein expression of phospho-AMPKα/AMPKα
6
5
4
3
2
1
0
Control (n=6)
Metformin (n=6)
B
i
ns
Relative expression of e-NOS/GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
Control (n=10)
Metformin (n=12)
ii
*
Relative expression of phospho-eNOS/GAPDH
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control (n=11)
Metformin (n=11)
iii
*
Protein expression of phospho-eNOS/e-NOS
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control (n=9)
Metformin (n=11)

Figure R4.3. The effect of metformin on protein expression detected by chemiluminescent Western blotting. A: Representative immunoblots of AMPKα, phospho-AMPKα, total e-NOS, phospho-eNOS (Ser^{1177}) and GAPDH expression. A: i) Results of relative densitometry analysis of AMPKα protein expression in the spleen relative to GAPDH expression, ii) Results of relative densitometry analysis of phospho-AMPKα protein expression in the spleen relative to GAPDH expression, iii) Results of relative densitometry analysis of phospho-AMPKα protein expression relative to total AMPK expression in the spleen. B: i) Results of relative densitometry analysis of e-NOS protein expression relative to GAPDH expression in the in the ischaemic gastrocnemius muscle, ii) Results of relative densitometry analysis of phospho-eNOS protein expression relative to GAPDH expression in the ischaemic gastrocnemius muscle, iii) Results of relative densitometry analysis of phospho-eNOS protein expression relative to total e-NOS expression in the ischaemic gastrocnemius muscle. Original blots presented below. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared between groups with Mann Whitney U test, * indicates $p<0.05$, ns indicates $p>0.05$.

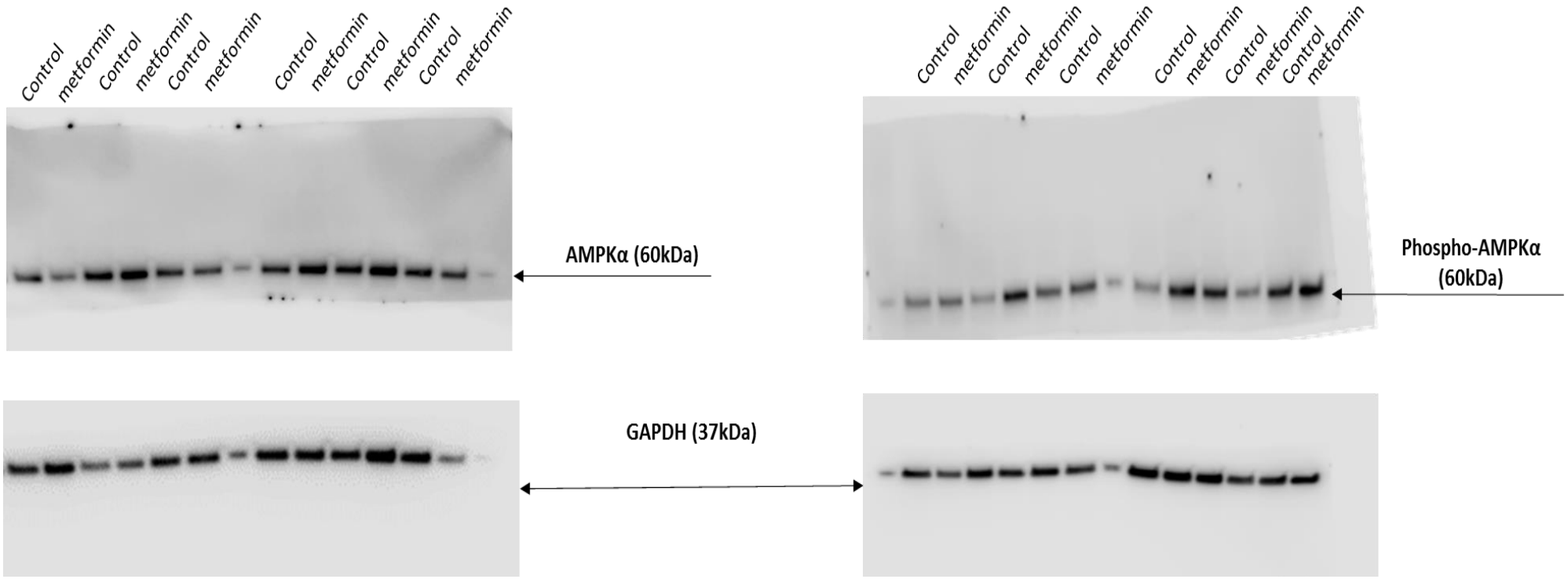


Figure R4.3C. Western blot images of AMPKα and phospho-AMPKα expression with their respective GAPDH blots for the effect of metformin on limb ischaemia. Unlabelled lanes contained ladders.

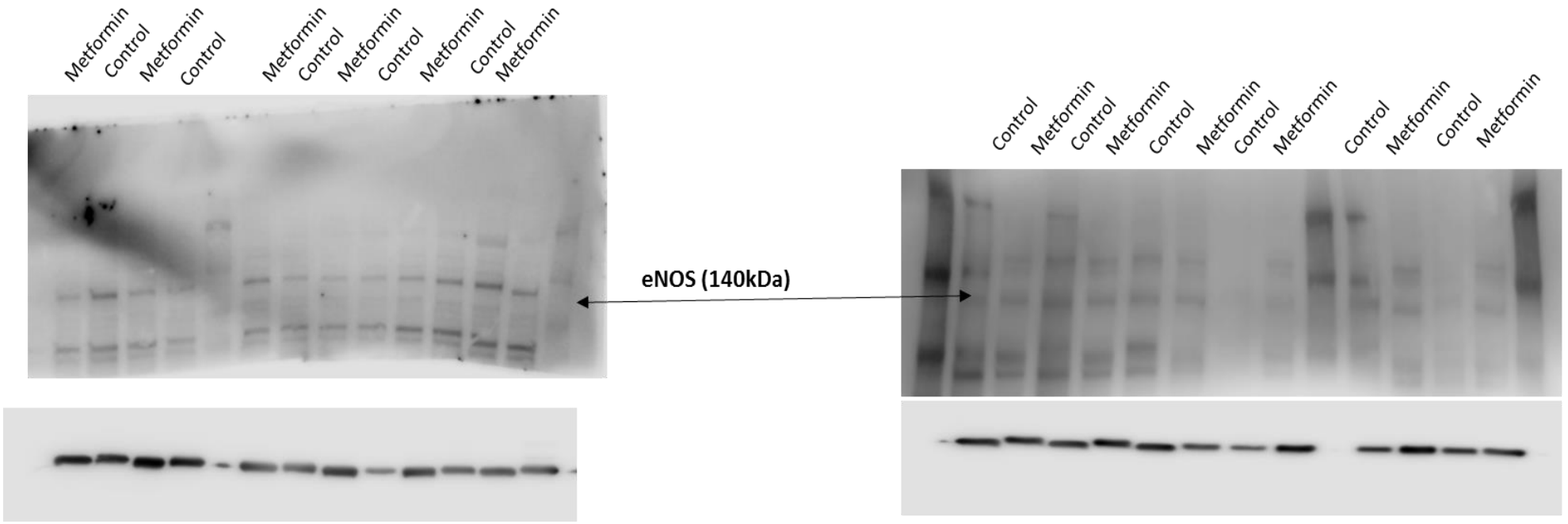


Figure R4.3D. Western blot images of e-NOS expression with their respective GAPDH blots. Unlabelled lanes contained ladders.

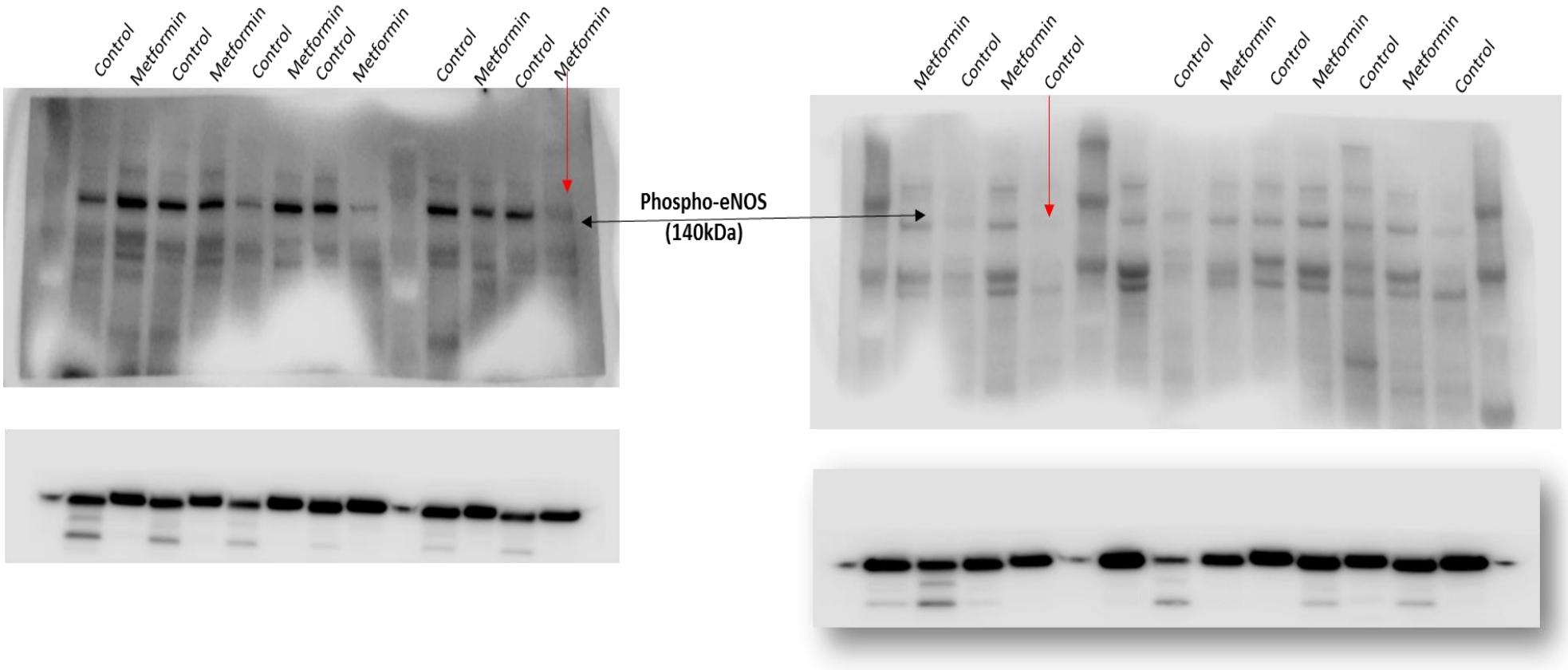


Figure R4.3E. Western blot images of phospho-eNOS expression with their respective GAPDH blots. Red arrows indicate samples excluded due to signal intensity being not quantifiable. Unlabelled lanes are contained ladders.

4.3.4 Circulating nitric oxide was increased in mice receiving metformin

Plasma nitrate was measured using a colourimetric assay (Cayman Chemicals) 4 weeks after administering metformin or vehicle. Plasma NO levels were greater in mice receiving metformin than controls (8.44±2.02 μM and 3.77±0.50 μM respectively, *p=0.024*; Figure R4.4B). Furthermore, non-fasting plasma glucose concentrations after 4 weeks of interventions were similar (*p=0.119*) in mice receiving metformin (14.25± 2.524 mmol/l) and vehicle (15.79± 3.25 mmol/l) (Figure R4.4B).

4.3.5 TXNIP was downregulated and PGC1α was upregulated in ischaemic muscle of mice receiving metformin

Relative protein and gene expression of the oxidative stress augmenting protein TXNIP was significantly downregulated (p=0.038, Figure R4.5B and p=0.017 respectively, Figure R4.6C) in mice receiving metformin compared to mice receiving vehicle control. Relative protein and gene expression of the mitochondrial biogenesis marker PGC1α, was significantly upregulated (*p=0.026*, Figure R4.5C and *p=0.028*, Figure R4.6D respectively) in the ischaemic gastrocnemius muscles of mice receiving metformin compared with controls.

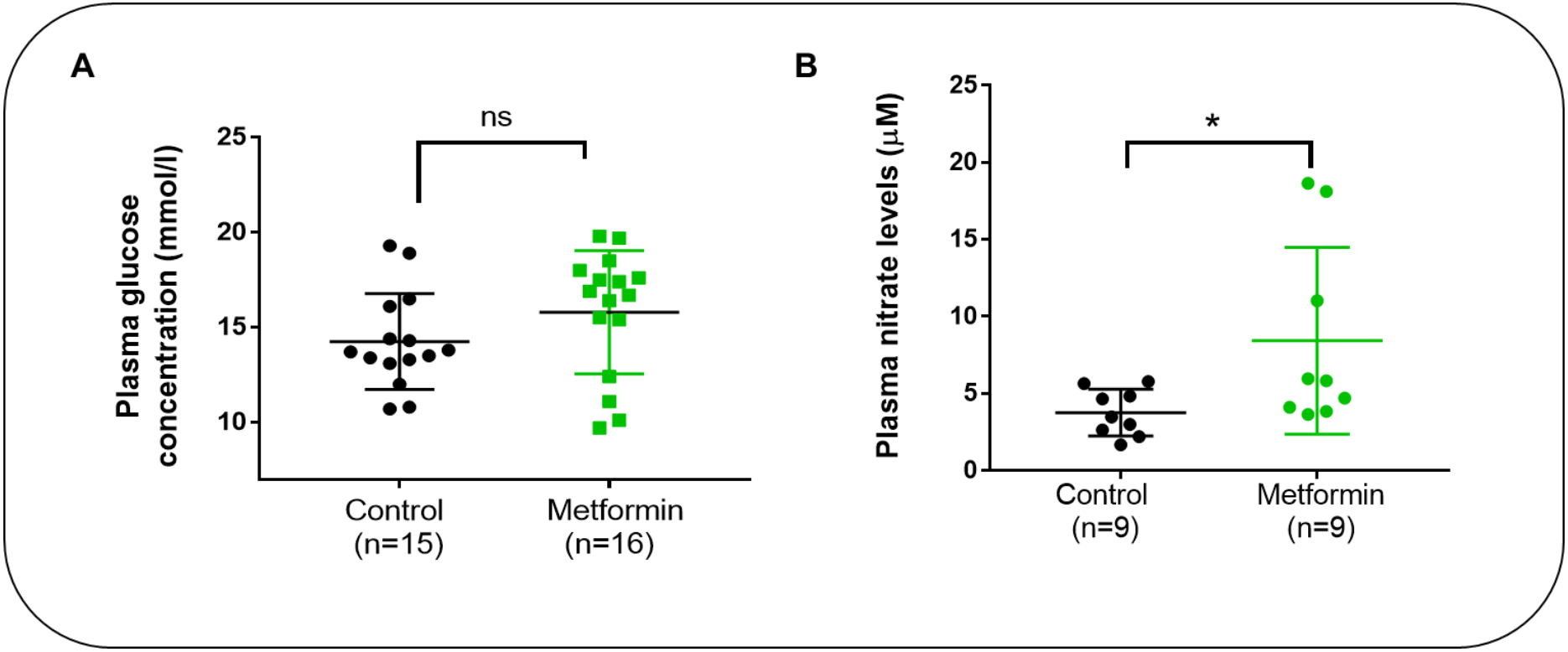


Figure R4.4. Effect of metformin on circulating plasma glucose and plasma nitrate levels. Plasma levels of glucose (A) and nitrate (B) 4 weeks after vehicle or metformin administration. Nitrate levels were determined using a colourimetric assay (Cayman Chemicals) and nitrate levels were considered as the index of NO levels. Data expressed as mean and SD and were compared between the groups using Mann Whitney U test, $p<0.05$ indicated by * and $p>0.05$ indicated as ns.

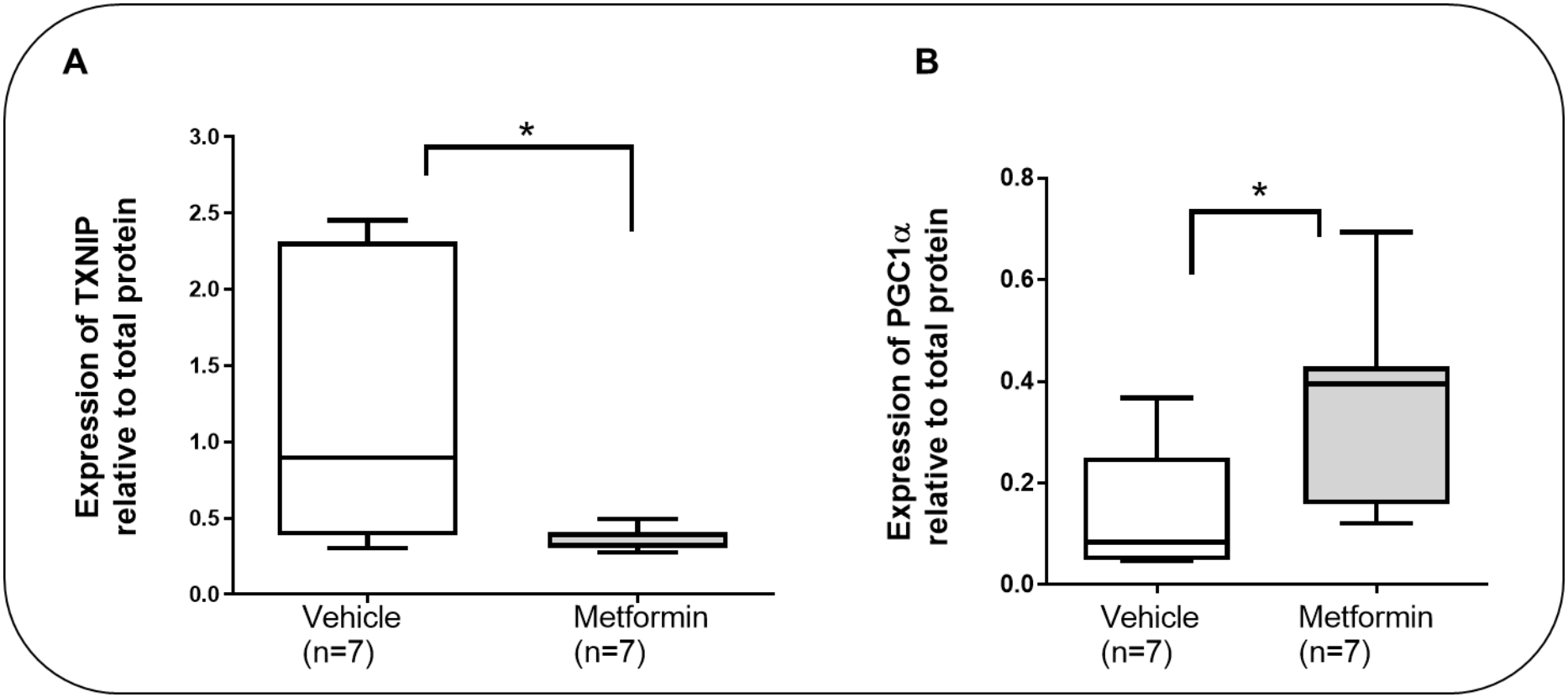


Figure R4.5. The effect of metformin on protein expression in the ischaemic gastrocnemius muscles detected by infrared fluorescent Western blotting. A: Results of relative densitometry analysis of PGC1α protein relative to total protein expression. B: Results of relative densitometry analysis of TXNIP protein relative to total protein expression. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared between groups with Mann Whitney U test, * indicates $p<0.05$, ns indicates $p>0.05$.

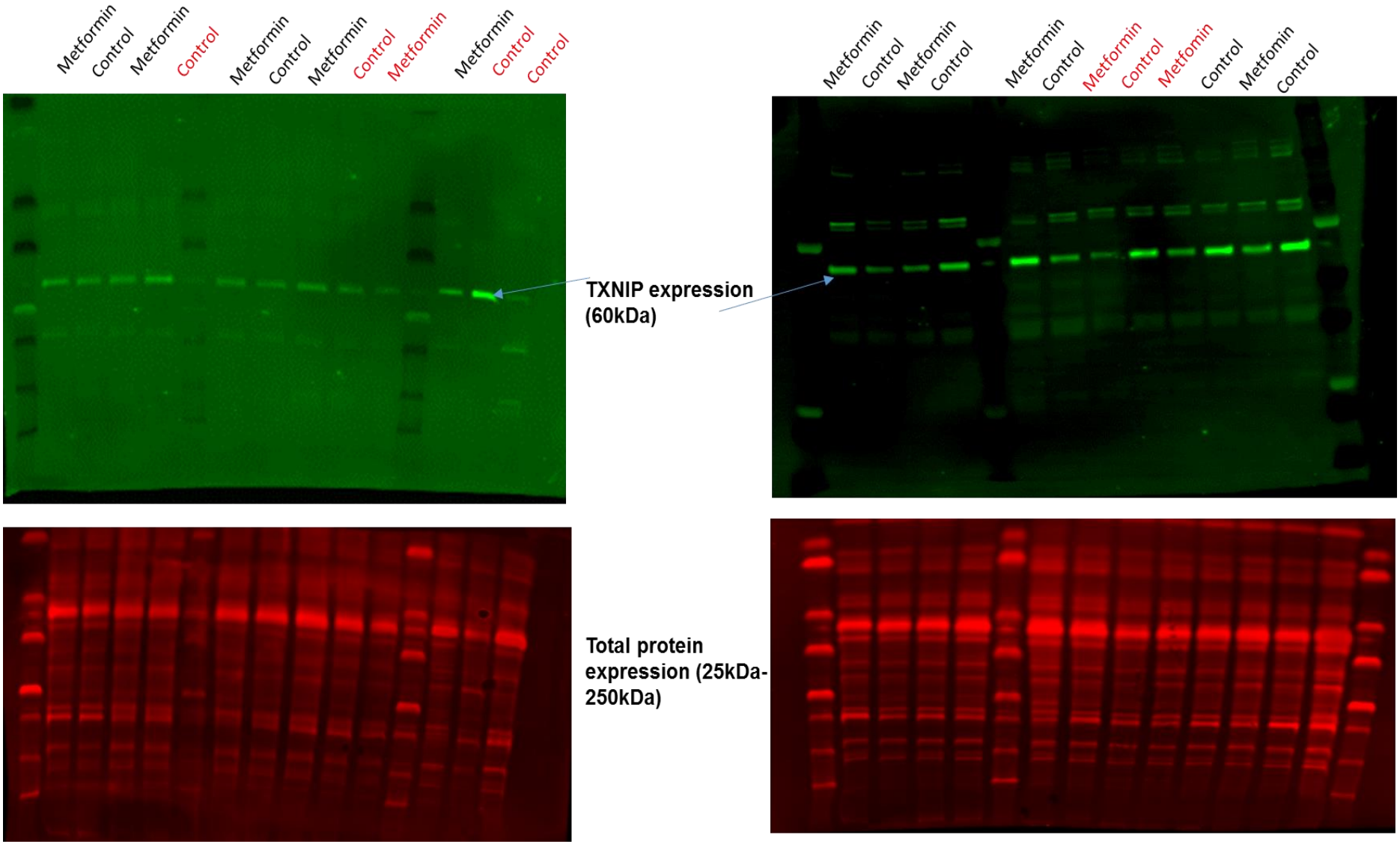
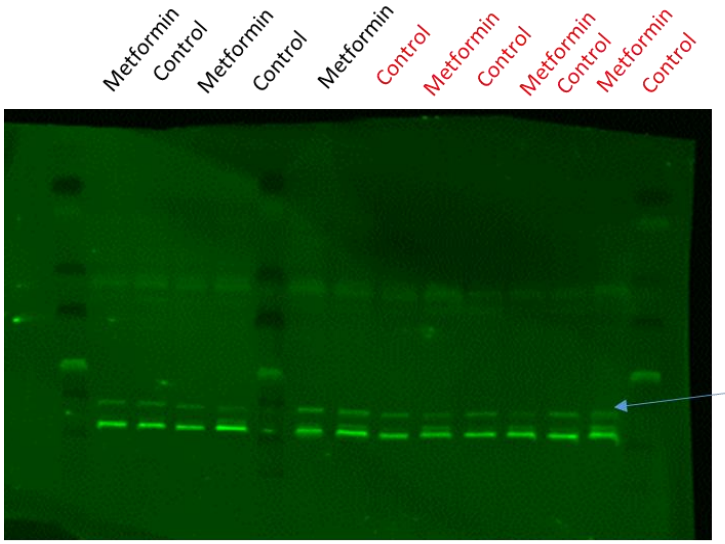
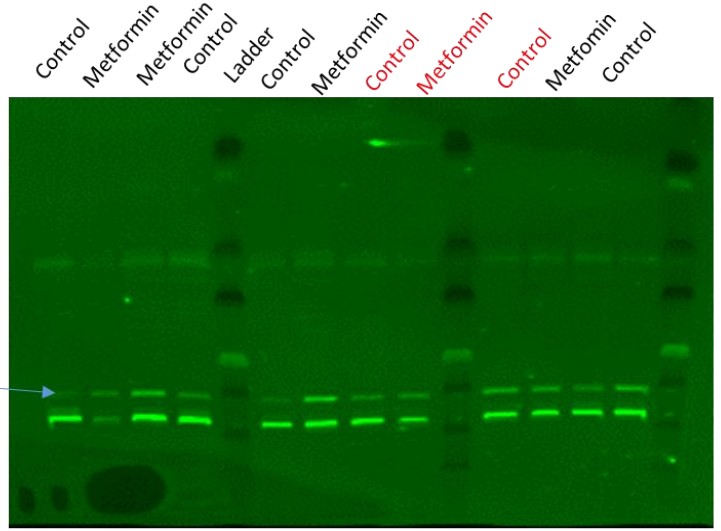
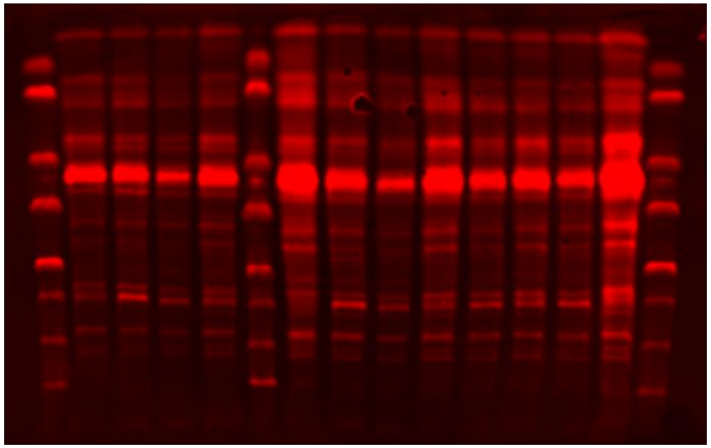

Metformin
Control
Metformin
Control
Metformin
Control
Metformin
Control
Metformin
Metformin
Control
Control
Metformin
Control
Metformin
Control
Metformin
Control
Metfomin
Control
Metfomin
Control
TXNIP expression (60kDa)
Total protein expression (25kDa-250kDa)

Figure R4.5C. Western blot images of TXNIP expression with their respective total protein expression blots. Red labels indicate samples excluded due to artefacts in lane interfering with quantitation of signal intensity.



PGC1a expression (90 kDa)





Total protein expression (25kDa-250kDa)

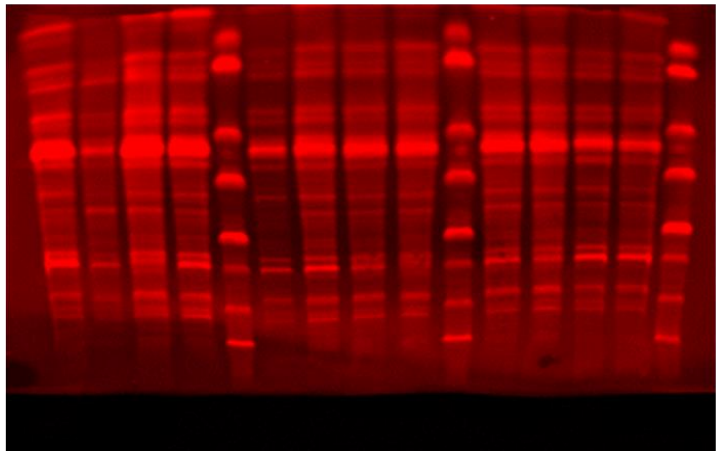


Figure R4.5D Western blot images of PGC1α expression with their respective total protein expression blots. Red labels indicate samples excluded due to artefacts in lane interfering with quantitation of signal intensity.

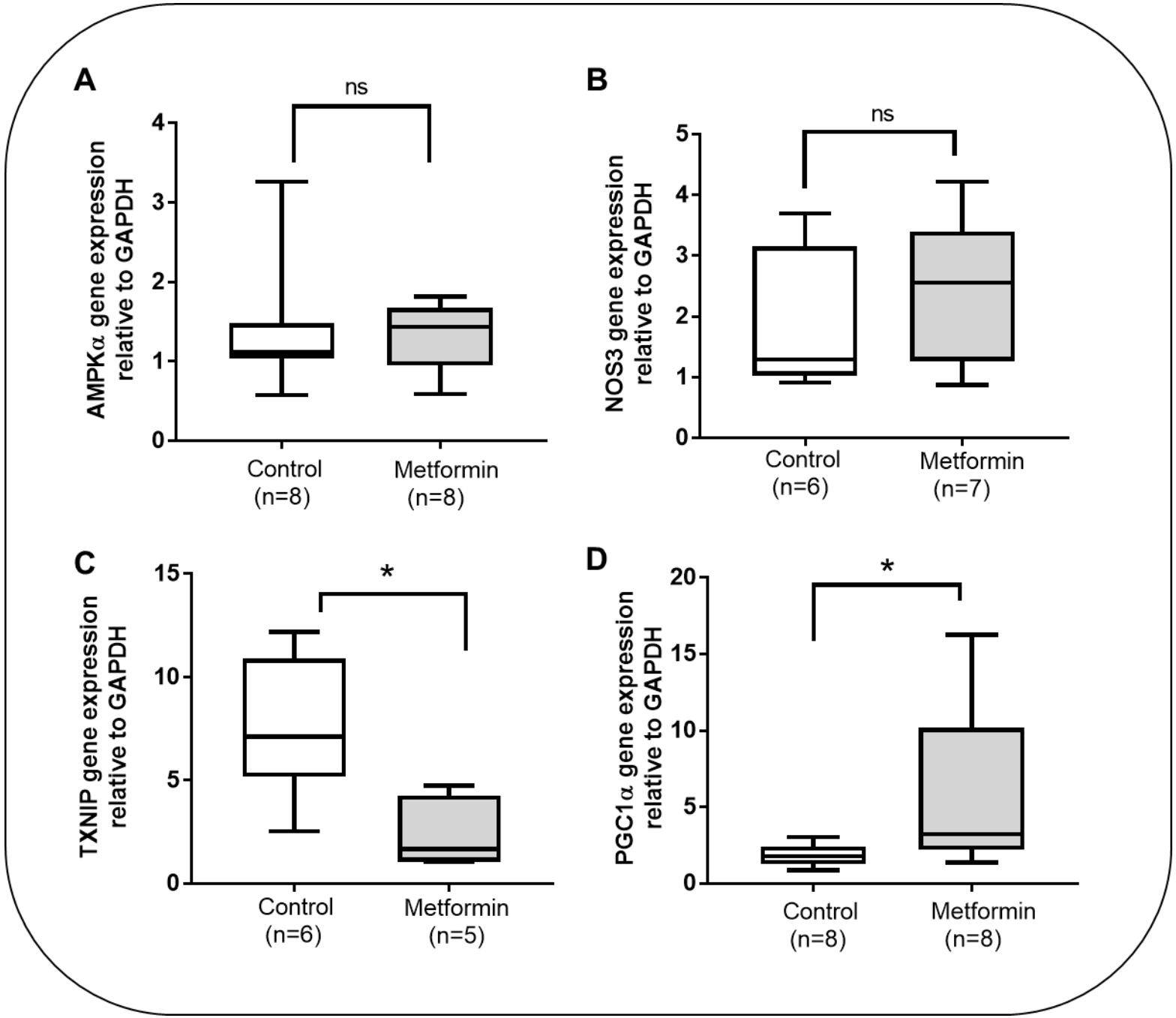

A
ns
AMPKα gene expression relative to GAPDH
0
1
2
3
4
Control (n=8)
Metformin (n=8)
B
ns
NOS3 gene expression relative to GAPDH
0
1
2
3
4
5
Control (n=6)
Metformin (n=7)
C
*
TXNIP gene expression relative to GAPDH
0
5
10
15
Control (n=6)
Metformin (n=5)
D
*
PGC1α gene expression relative to GAPDH
0
5
10
15
20
Control (n=8)
Metformin (n=8)

Figure R4.6. Effect of metformin on mRNA expression in the ischaemic gastrocnemius muscles determined by qRT-PCR. A: Results of AMPKα1 mRNA expression relative to GAPDH, B: Results of Nos3 mRNA expression relative to GAPDH, C: Results of TXNIP mRNA expression relative to GAPDH and D: Results of PGC1α mRNA expression relative to GAPDH. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared between groups with Mann Whitney U test, * indicates $p<0.05$, ns indicates $p>0.05$.

4.4 Discussion

The main outcomes of this study were:

1. Metformin administration promoted recovery of hindlimb blood supply in the novel mouse model of ongoing ischemia.

2. Metformin intervention resulted in upregulation of AMPKα activity and e-NOS activity with increased circulating NO levels. Additionally, decreased gene and protein expression of oxidative stress marker TXNIP and increased gene and protein expression of the mitochondrial biogenesis marker PGC1α were indicated in the ischaemic tissues of mice receiving metformin.

3. Metformin intervention did not promote hindlimb function assessed by treadmill walking distance in the two-stage model of HLI.

HLI was achieved in this study using a sequential two step surgical procedure reported in Chapter 3. The improvement in blood supply in mice receiving metformin is supportive of previous similar findings in the one stage HLI model and two uncontrolled studies in PAD patients (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992, Takahashi, Shibata et al. 2015). In the present study, the improvement in blood supply to the ischaemic limbs by metformin was mild within a period of 4 weeks.

Indirect activation of AMPKα is intimately associated with the pleiotropic actions of metformin (Foretz, Guigas et al. 2014, Langone, Cannata et al. 2014, Kinaan, Ding et al. 2015). This activation is a result of a mild and transient inhibitory effect by metformin on the mitochondrial complex 1 of the electron transport chain (Foretz, Guigas et al. 2014). AMPKα activity was upregulated in mice receiving metformin suggested by the increased protein expression of phospho-AMPKα to total-AMPKα, suggesting that recovery of blood supply from an established steady state of ischaemia was improved via AMPKα dependent mechanisms. AMPKα gene expression was similar in mice receiving metformin compared to mice receiving vehicle control indicating AMPKα protein activity rather than gene expression is modulated by metformin. Among possible downstream signals implicated in the improvement in blood supply by metformin through AMPKα, this study investigated e-NOS, PGC1α, and TXNIP which are associated in the modulation of angiogenesis/arteriogenesis, oxidative stress and mitochondrial biogenesis respectively. Activation of AMPKα by metformin has been demonstrated to lead to phosphorylation of e-NOS resulting in increased NO bioavailability, which could subsequently promote revascularisation (Rena, Pearson et al. 2013, Takahashi,

Shibata et al. 2015). It has been previously reported that metformin does not promote recovery of blood supply in e-NOS deficient mice in which HLI is induced with the one-stage approach (Takahashi et al., 2015). These findings strongly suggest that e-NOS is a key mediator of the action of metformin on limb reperfusion (Takahashi, Shibata et al. 2015). In the current study, we found that the protein levels of phospho-eNOS to total e-NOS were upregulated within the ischaemic gastrocnemius muscles and plasma nitrate levels were increased in mice receiving metformin but not the gene expression of Nos3. As a result, plasma NO levels were higher in mice receiving metformin. This suggests metformin promoted increased e-NOS activity as in the previous acute HLI study (Takahashi, Shibata et al. 2015).

Endothelial cell migration and proliferation are key processes in revascularisation (Goveia, Stapor et al. 2014). The endothelial-driven proliferative response requires upregulation of mitochondrial mass and function (Tang, Luo et al. 2014). Such mitochondrial biogenesis requires the coordinated replication and expression of mitochondrial DNA with the parallel expression of nuclear-encoded mitochondrial genes (Uittenbogaard and Chiaramello 2014). A key regulator of this process is PGC-1α (Uittenbogaard and Chiaramello 2014). PGC-1α promotes acceleration of angiogenesis by endothelial cells through mitochondrial biogenesis which meets endothelial cell metabolic demands during proliferation (Rowe, Jiang et al. 2010). Metformin has been previously reported to upregulate PGC1α in skeletal muscles through AMPKα stimulation (Jager, Handschin et al. 2007, Fernandez-Marcos and Auwerx 2011). The results of the present study indicate that PGC1α protein and gene expression were upregulated in the ischaemic gastrocnemius muscles of mice receiving metformin leading to improved blood supply.

Oxidative stress is known to impair endothelial function and is associated with impairment of revascularisation (McDermott 2015). Thioredoxin is an antioxidant protein which regulates the redox response to oxidative stress by maintain the reducing environment of tissues (Nishiyama, Matsui et al. 1999). TXNIP is a negative regulator of thioredoxin and has been shown to have adverse effects on ischemia and oxidative stress (Dunn, Buckle et al. 2010). TXNIP knockdown has been shown to rescue blood-flow impairment and improve functional recovery in ischaemic hindlimbs (Dunn, Simpson et al. 2014). Normalisation of hyperglycemia-induced TXNIP expression to non-diabetic levels has been reported to rescue diabetes-related impairment of ischemia-mediated angiogenesis (Dunn, Simpson et al. 2014). In addition, TXNIP has been shown to enhance ischemia-reperfusion injury in response to acute hyperglycemia (Yoshioka, Chutkow et al. 2012). TXNIP knockdown is associated with

increased e-NOS expression and NO production (Wu, Zheng et al. 2013, Dunn, Simpson et al. 2014). In ischemia/reperfusion injury, TXNIP is associated with harmful consequences by promoting oxidative damage in tissues via its pro-oxidative effects (Lane, Flam et al. 2013). Metformin has been reported to reduce the expression of TXNIP through AMPKα activation in aortic endothelial cells (Li, Kover et al. 2015). In the current study, it was found that TXNIP protein and gene expression was downregulated in the ischaemic gastrocnemius muscles of mice receiving metformin.

Furthermore, in this study a non-fasting impairment of blood glucose concentrations was not observed in mice receiving metformin for 4 weeks at the daily human equivalent dosage of 2.0g/80 kg person. This suggests that metformin could be tolerated by non-diabetic subjects without experiencing adverse hypoglycaemic episodes.

In this study, the effect of metformin intervention on treadmill walking performance to defined pain was examined. Unlike the improvement in blood supply, there was no improvement in walking capacity in mice receiving metformin within the experimental period. It was assumed in this study that the frequency of visits to the rest zone by mice on the treadmill belt is reflective of lower limb discomfort, such as that experienced during claudication in patients with PAD. Therefore, fatigue was defined in this study as 10 visits to the rest zone despite electrical stimulus for encouraging the mice to walk on the treadmill. A confounding variable of this method is that mice exhibit different running styles with some styles which cause premature stopping before discomfort by pain is achieved (Marcinko, Bujak et al. 2015). In addition, no tissue injury or tissue loss was evident in the experimental mice suggesting the symptomatic severity of ischaemia in the experimental model was mild and may be representative of intermittent claudication patients' symptoms. Additional possible limitations could be the length of time on intervention to derive functional benefits, dose and sample size. The mice were only assessed for 4 weeks of metformin intervention which may be short for functional benefits to appear. Length of time for improvement to be apparent is a feature of current management strategies for PAD such as with supervised walking exercise interventions which typically require months before substantial improvement in walking performance is realised (McDermott 2013). The dose used in this study was the currently prescribed human dose for managing hyperglycaemia in diabetes patients. In this study the improvement in blood supply by metformin was only observed 2 weeks after commencing the intervention. This suggests that examining a higher dose at the start of intervention may be relevant for faster improvement of blood supply by metformin intervention. Another limitation of the present

study for determining the treadmill walking performance of the ischaemic mice receiving metformin or vehicle is the sample size. Sample size for this study was estimated for the outcome measure of LDI. The present study is limited in sample size to derive a clear conclusion of the effect of metformin intervention on treadmill walking capacity in the experimental model of established ongoing unilateral limb ischaemia.

Inhibition of complex 1 of the mitochondrial respiratory chain resulting in AMPKα activation causing downstream signals is reported to be the main mechanism of action of metformin in order to have its effects. A recently described AMPK activator, R419, activates AMPK by inhibiting the mitochondrial complex 1 similar to metformin (Marcinko, Bujak et al. 2015). R419, has been shown to improve treadmill running capacity by over 30% in obese mice fed a high-fat diet (Marcinko, Bujak et al. 2015). Metformin's ability to inhibit complex 1 has been reported to be mild and transient (Foretz, Guigas et al. 2014). Therefore, a strong inhibitor of complex 1 resulting in AMPK activation may generate more favourable blood supply improvements and functional improvements in HLI.

In summary, the findings of this study suggest that metformin intervention at the currently prescribed human relevant dose improves blood supply to a state of established ongoing experimental limb ischaemia. The improvement in blood supply was characterised to be a result of the upregulation of AMPKα activity, associated with increased activation of e-NOS in the ischaemic muscles, increased bioavailability of circulating NO, increased expression of PGC1α and reduced expression of TXNIP. Based on previous reports these changes would be expected to improve endothelial function by enhancing mitochondrial biogenesis (Jager, Handschin et al. 2007, Fernandez-Marcos and Auwerx 2011), lowering oxidative stress and improving NO bioavailability through upregulated activity of e-NOS (Wu et al., 2013), consequently improving blood supply to ischaemic muscles. Limb impairment was not suggested to be improved by metformin intervention at the currently prescribed human dose within the experimental period and the limited sample size examined.

This study has numerous strengths and weakness. Strengths include the use of a pre-clinical model with relevant pathophysiological features of human limb ischemia and use of relevant drug dose and prospective study design with pre-established sample size calculations. The relevance of pre-clinical animal models to assess therapies for PAD is controversial (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Although a single approach in animals cannot fully recapitulate the human condition, an *in vivo*

model which may recapitulate the salient features of the disease will potentially aid in drug discovery. Limitations for the current study include the lack of a comprehensive investigation to explore the underlying mechanisms by which metformin improves limb blood supply including measure angiogenesis, oxidative stress or mitochondrial biogenesis as possible mechanisms of benefit for metformin and the relatively short monitoring of the outcome, although 4 weeks is typical of studies within mouse models. Only one dose of metformin was examined in this study, although relevant to the currently prescribed human daily dose for managing hyperglycaemia, higher doses may need to be explored for improving blood supply faster. The experimental model used in the current study also has mild tissue injury and degree of functional impairment is mild. Additional relevant models with increased severity of tissue injury and ambulatory impairment may need to be developed and explored for the potential of metformin for translation.

In conclusion, the findings of this pre-clinical study highlight the therapeutic potential of metformin for improving limb blood supply from an established ongoing state of ischemia. Cautious optimism should be exercised when translating these findings as longer treatment times may be needed in clinical trials before observing any improvements to realise potential of metformin for managing PAD symptoms. In this pre-clinical study 3-4 weeks of metformin administration was necessary before improvement in blood supply was initially observed.

Chapter 5
The effect of Angiotensin converting enzyme 2 deficiency in experimental limb ischaemia

5.1 Introduction

In Chapter 3 of this thesis, a new mouse model was developed in which HLI was induced through a two-stage procedure in order to better simulate limb ischemia in PAD patients. This approach led to a sustained state of severe ischemia that was prolonged over 4 weeks in adult *ApoE*$^{-/-}$ mice. This novel mouse model was used in the current study.

Effective pharmacological treatments to restore blood supply and treat the leg symptoms of PAD are an unmet medical need (Vemulapalli, Dolor et al. 2015). NO is an important promoter of angiogenesis and arteriogenesis for post-ischaemic recovery and NO is primarily derived from e-NOS activity (Yu, deMuinck et al. 2005). Endogenous compounds which modulate e-NOS activity could play important roles in recovery of blood supply to limb ischaemia and e-NOS could be a potential target for drug development in PAD (Forte, Conti et al. 2016). Activity of e-NOS to enhance NO bioavailability could be promoted through phosphorylation at serine 1177 and by limiting e-NOS uncoupling (Forte, Conti et al. 2016). Evidence suggests angiotensin converting enzyme 2 (ACE2) could be an important modulator of e-NOS activity (Zhang, Wang et al. 2014). ACE2 catalyses the conversion of angiotensin II (Ang II) to angiotensin-(1–7) (Ang-(1–7)) (Crackower, Sarao et al. 2002, Rabelo, Todiras et al. 2016, Santos, Sampaio et al. 2018). Ang 1-7 signalling promotes e-NOS activity through phosphorylation via Akt (Sampaio, Souza dos Santos et al. 2007, Patel and Schultz 2013). In addition, Ang II is known to promote e-NOS uncoupling to produce superoxide than NO. Degradation of Ang II by ACE2 limits uncoupling of e-NOS promoting NO bioavailability and e-NOS activity (Yamamoto, Ohishi et al. 2006, Alghamri, Weir et al. 2013, Moritani, Iwai et al. 2013).

Genetic deficiency of ACE2 (*ACE2*$^{-/y}$) in mice results in low tissue and circulating levels of Ang 1–7 and higher Ang II levels (Thomas, Pickering et al. 2010, Bernardi, Burns et al. 2012, Tikellis, Pickering et al. 2012, Wysocki, Ortiz-Melo et al. 2014). *ACE2*$^{-/y}$ mice have also been reported to demonstrate drastic reduction in e-NOS expression at both protein and mRNA levels, and a decrease in NO concentrations (Yamamoto, Ohishi et al. 2006, Rabelo, Todiras et al. 2016). Despite evidence suggesting potential of ACE2 to play an important role in modulating Ang1-7 and NO levels, the role of ACE2 in limb ischaemia is unknown. Furthermore, ACE2 activity has been suggested to be differentially regulated in males and females (Soler, Riera et al. 2012, Soro-Paavonen, Gordin et al. 2012), with serum ACE2

activity higher in males than females (Úri, Fagyas et al. 2016). Thus, this study investigated the effect of *ACE2*$^{-/y}$ on experimental two-stage HLI in both male and female *ApoE*$^{-/-}$ groups.

The hypothesis and aim of this study were:

Hypothesis:

ACE2$^{-/y}$ mice would have more severe limb ischaemia than sex and age matched ACE2 present control mice 4 weeks after HLI induction.

Aim:
To examine the effect of *ACE2*$^{-/y}$ on two-stage HLI induction in adult mice.

5.2 Methods

5.2.1 Ethics approval and mouse husbandry

Institutional ethics was obtained for the studies presented in this Chapter (Appendix) and mice were maintained as described in General Methods (Chapter 2.1). Male and female apolipoprotein E null (*ApoE*$^{-/-}$) mice (C57BL/6J background) aged 6 months were obtained from the Animal Resource Centre, Canning Vale, Western Australia. Genotyped male and female *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice were provided by Associate Professor Chris Tikellis at Baker IDI, Melbourne, Australia. Mice were housed and maintained as detailed in Chapter 2.

5.2.2 Study design

This study was designed to compare the effect of *ACE2*$^{-/y}$ on the severity of HLI in male and female *ApoE*$^{-/-}$ mice. Six-month-old male *ApoE*$^{-/-}$ (n=8), 6-month-old male *ACE2*$^{-/y}$*ApoE*$^{-/-y}$ mice (n=8) 6-month-old female *ApoE*$^{-/-}$ (n=11), and 6-month-old female *ACE2*$^{-/y}$*ApoE*$^{/-y}$ mice (n=10) were employed in this study. HLI was induced by the two-stage method and the limb blood supply was assessed as the primary outcome measure by LDI. Secondary outcome measures were ambulatory function by Tarlov scoring and tissue injury assessed by ischaemia scoring (Figure M5.1).

5.2.3 Sample size

The sample size for this study was estimated for the primary outcome measure of LDI. The expected LDI outcome for the *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice was calculated using LDI data from the

two-stage HLI group of mice developed in chapter 3. In the previous study 10 *ApoE*$^{-/-}$ mice were imaged 28 days after ischaemia induction and mean (SD) flux ratio was 0.54 (0.093). It was assumed that in the *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice ischaemic hind limb blood supply would be decreased by 25%, which is suggested to cause limb impairment and tissue injury (Silvestre, Bergaya et al. 2001, Zhu, Zhang et al. 2016). Based on these assumptions and aiming to achieve an 80% power with an alpha of 0.05 an estimated minimum of 8 mice were needed per group. Eight to 11 mice were used per group based on availability to account for any exclusions which may have arisen from technical difficulties.

5.2.4 Hindlimb ischaemia induction

Unilateral HLI was induced in the left hindlimb of each mouse through the two-step surgical procedure described in Chapter 2.

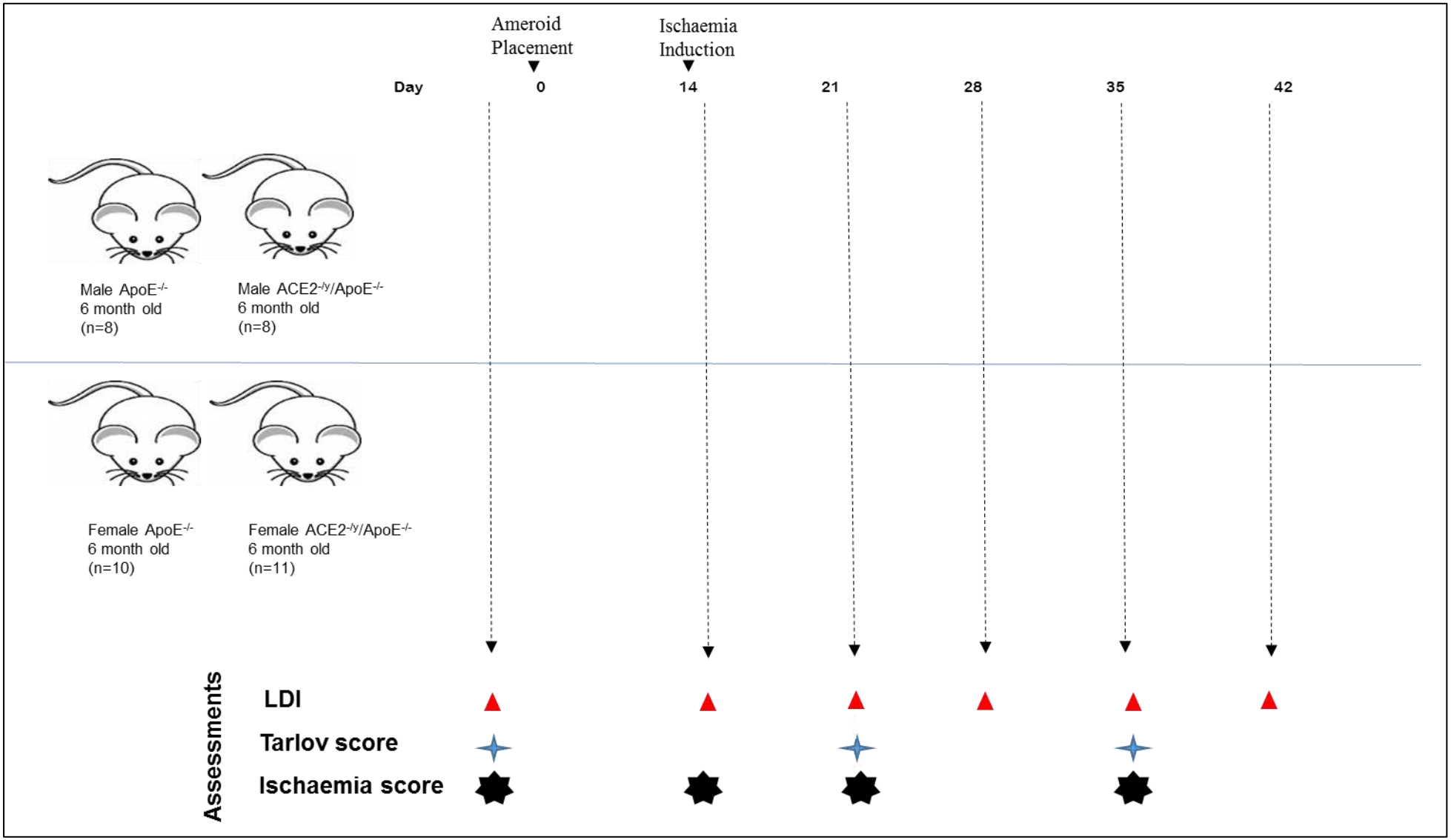

Ameroid Placement
Ischaemia Induction
Day
0
14
21
28
35
42
Male ApoE-/-
6 month old
(n=8)
Male ACE2-/y/ApoE-/-
6 month old
(n=8)
Female ApoE-/-
6 month old
(n=10)
Female ACE2-/y/ApoE-/-
6 month old
(n=11)
Assessments
LDI
Tarlov score
Ischaemia score

Figure M5.1. Study design: The effect of *ACE2*$^{-/y}$ on the severity of HLI in male and female *ApoE*$^{-/-}$ mice. Limb blood supply was assessed by LDI, ambulatory function and tissue injury were assessed by the Tarlov score and ischaemia score, respectively. Ischaemia was established by the two-stage HLI induction method, initial ameroid placement on the FA on day 0 and ligation and excision of FA on day 14.

5.2.5 Laser Doppler imaging

LDI measurements were carried out pre-operatively (baseline) and on days 7, 15, 21, 28, 35 and 41 after ameroid placement. (Fig M5.1). LDI measurements were performed as detailed in Chapter 2.

5.2.6 Observational functional scoring and assessment of ischaemia

Observational assessment of limb function (Tarlov score) and ischaemia (Ischaemia score) were carried out and recorded pre-operatively (baseline), and on days 21 and 35 after commencing the experiments. Scoring was performed as detailed in Chapter 2.

5.2.7A Tail cuff plethysmography

Blood pressure (BP) was measured using a computerised, non-invasive, tail-cuff system (Kent Scientific, USA). Mice were restrained on clear acrylic restrainer with a nose cone and placed on a thermal pad at 37°C. The measurements were performed in a quiet environment and each mouse was acclimatised to the restrainer. The occlusion cuff was placed on the base of the tail and the volume pressure recorder cuff on the remaining end of the tail. The measurement device was turned on to commence continuous cycles of BP measurements until consistent readings were obtained. Displayed measurements were recorded and the average of three consecutive consistent readings were used for each mouse.

5.2.7B Repeatability of tail cuff plethysmography recordings

The intra-observer repeatability of the systolic blood pressure (SBP) measurement was performed on 10 separate male *ApoE*$^{-/-}$ mice without limb ischaemia. SBP was measured as described above and measurements were repeated again the next day. The intra-observer repeatability of the SBP measurement was CoV%=5.64% on 9 *ApoE*$^{-/-}$ mice assessed on 2 separate measurements (Appendix Table A).

5.2.8 Statistical analysis

Graphpad Prism V6.0 (GraphPad Software, San Diego, CA) and R studio software programs were used to analyse data. Graphpad was used to construct graphs. Data were tested for normality using D'Agostino-Pearson normality test. LDI data are expressed as mean±SEM. LME model analysis was used to compare LDI data between groups. For LME analyses variation between individual mice were treated as random effects. LDI data and time were treated as fixed effects. Model fit was assessed by examination of the spread of standardised residuals and qq-normal plots. Data were square root transformed to fit model assumptions. Interaction between time and groups were assessed by LME. Tarlov scores and ischaemia scores were expressed as mean and SEM and compared between groups by Mann-Whitney U test. SBP data were expressed as median and interquartile ranges and compared between groups by Mann-Whitney U test. All comparisons were considered significant at $p<0.05$.

5.3 Results

5.3.1 *ACE2$^{-/y}$ApoE$^{-/-}$* mice with two-stage HLI had comparable limb ischaemia and functional impairment to *ApoE$^{-/-}$* mice with two-stage HLI

Female *ACE2$^{-/y}$ApoE$^{-/-}$* mice and female *ApoE$^{-/-}$* mice had similar levels of hindlimb blood supply before ischaemia induction (Figure R5.1). Two-stage HLI induction in both groups resulted in a similar level of ischaemia (Figure R5.1 Female *ACE2$^{-/y}$ApoE$^{-/-}$* and *ApoE$^{-/-}$* mice had comparable limb blood supply over the experimental period (p=0.212; Figure R5.1).

Functional impairment assessed by Tarlov scores between female *ACE2$^{-/y}$ApoE$^{-/-}$* and female *ApoE$^{-/-}$* mice indicated that the similar impairment in limb function in *ACE2$^{-/y}$ApoE$^{-/-}$* and female *ApoE$^{-/-}$* groups over the course of the experiment (Figure R5.3A). Ischaemia scores in female *ACE2$^{-/y}$ApoE$^{-/-}$* and female *ApoE$^{-/-}$* mice were similar over the course of the experiment (Figure R5.4B)

Blood supply was similar between male *ACE2$^{-/y}$ApoE$^{-/-}$* mice and male *ApoE$^{-/-}$* mice at baseline (Figure R5.2) and the LDI ratio was similar in the *ACE2$^{-/y}$ApoE$^{-/-}$* and *ApoE$^{-/-}$* groups over 28 days after HLI induction (Figure R5.2). Blood supply over the experimental period was similar between male *ACE2$^{-/y}$ApoE$^{-/-}$* and *ApoE$^{-/-}$* mice (p=0.263; Figure R5.2).

Tarlov scores were similar between male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and male *ApoE*$^{-/-}$ mice over the course of the experiment (Figure 5.3B). Ischaemia scores were similar ischaemia scores between male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and male *ApoE*$^{-/-}$ mice (Figure 5.4A).

5.3.2 Systolic blood pressure were similar in *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice

SBP in female *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and female *ApoE*$^{-/-}$ mice was similar throughout the experiment (Figure R5.5A). SBP was similar in male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and male *ApoE*$^{-/-}$ mice throughout the experiment (Figure 5.5 B).

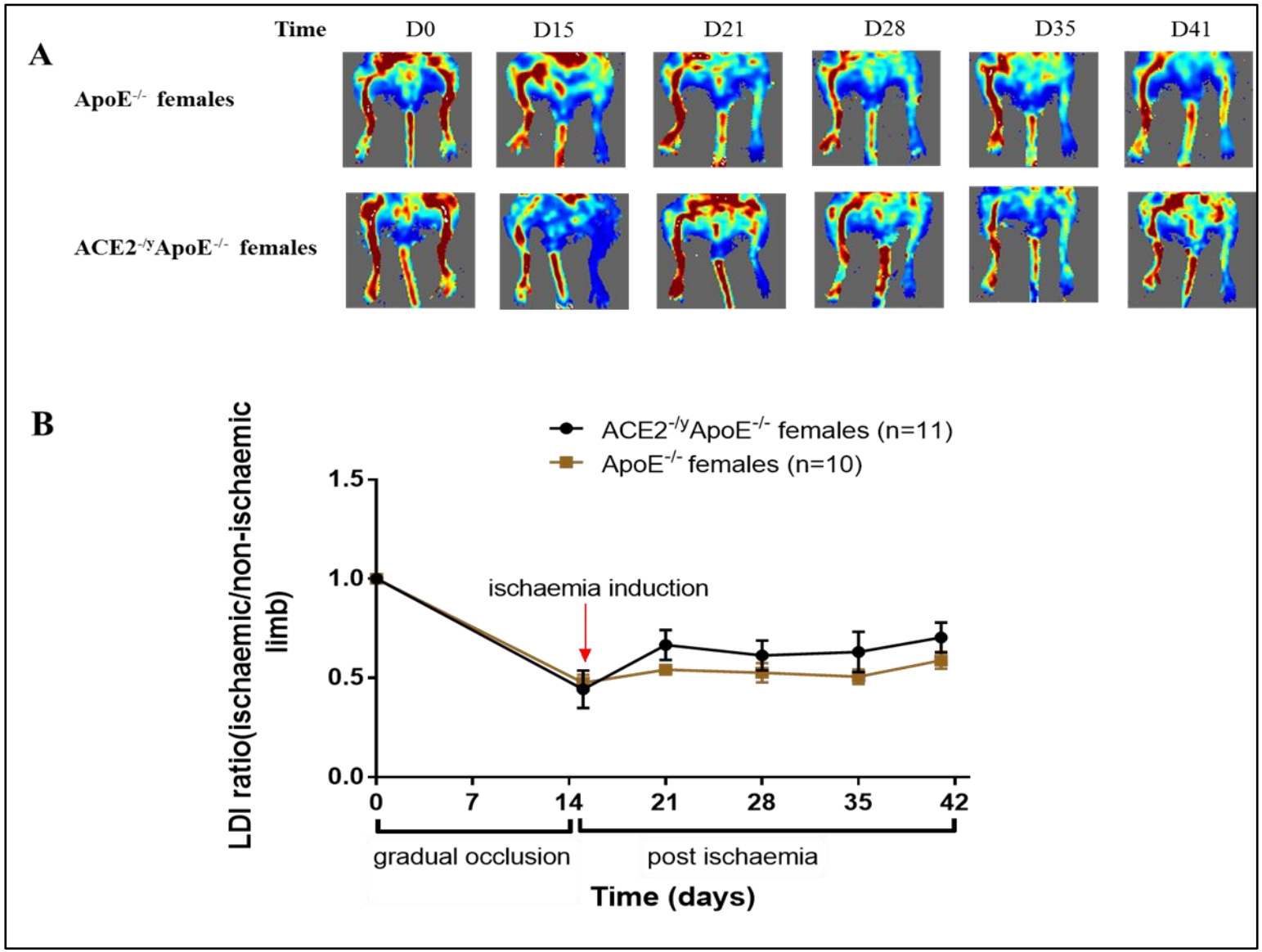


Figure R5.1: Effect of *ACE2*$^{-/y}$ on limb blood supply in female mice with two stage HLI. A: Representative laser Doppler images of female *ACE2*$^{-y}$-*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice. B: Laser Doppler imaging limb perfusion ratios of female *ACE2*$^{/y}$-*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice. LME analysis of post ischaemia period suggested no significant difference between the groups (p=0.212). Data expressed as mean ±SEM and the graph represented as LDI ratio relative to baseline values for each group.

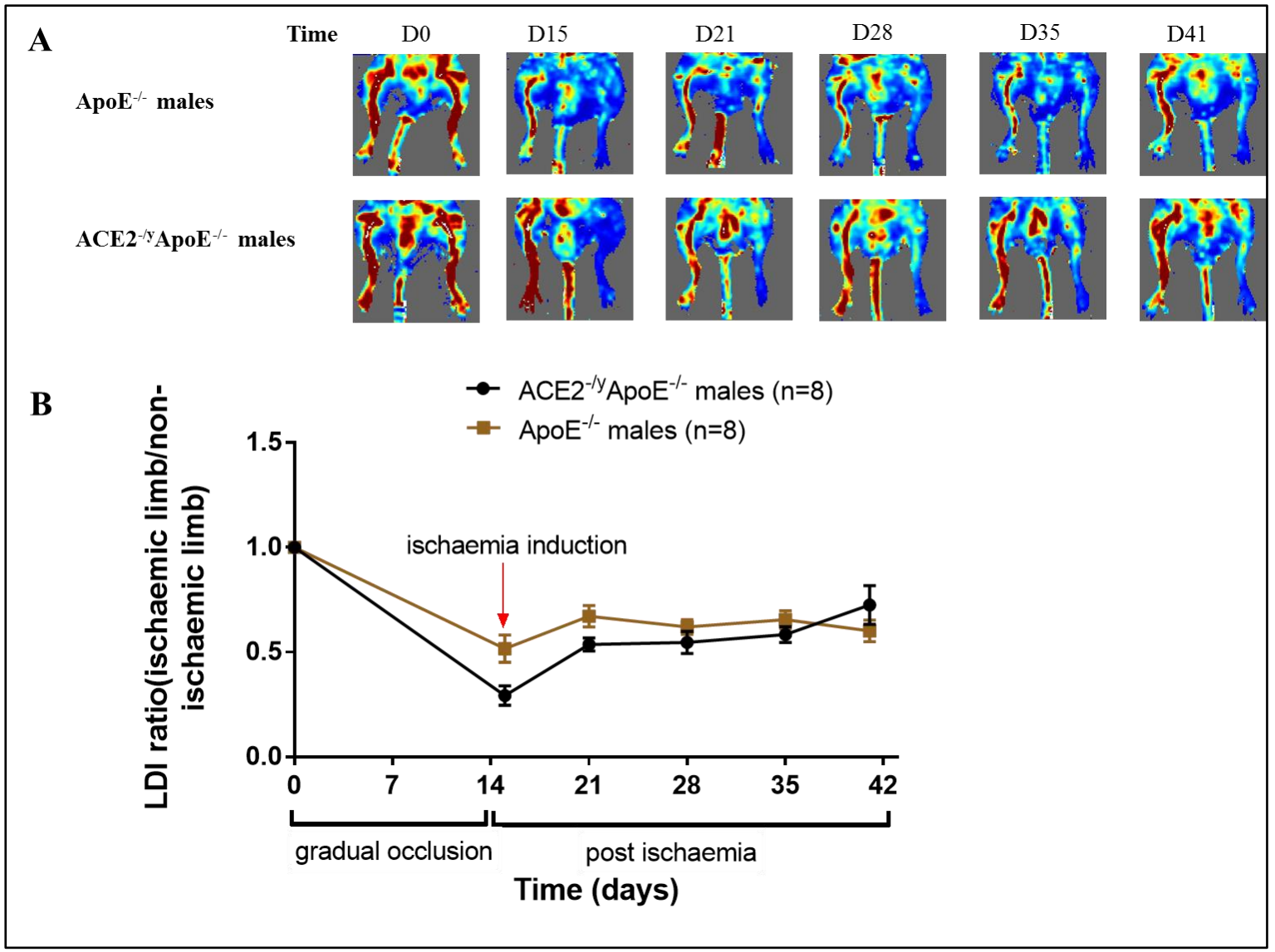


Figure R5.2. Effect of *ACE2*$^{-/y}$ on limb blood supply on male mice with two stage HLI. A: Representative laser Doppler images of male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice. B: Laser Doppler imaging limb perfusion ratios of male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice. LME analysis of post ischaemia period suggested no significant difference between the groups (p=0.263). Data expressed as mean ±SEM and graph represented as LDI ratio relative to baseline values for each group.

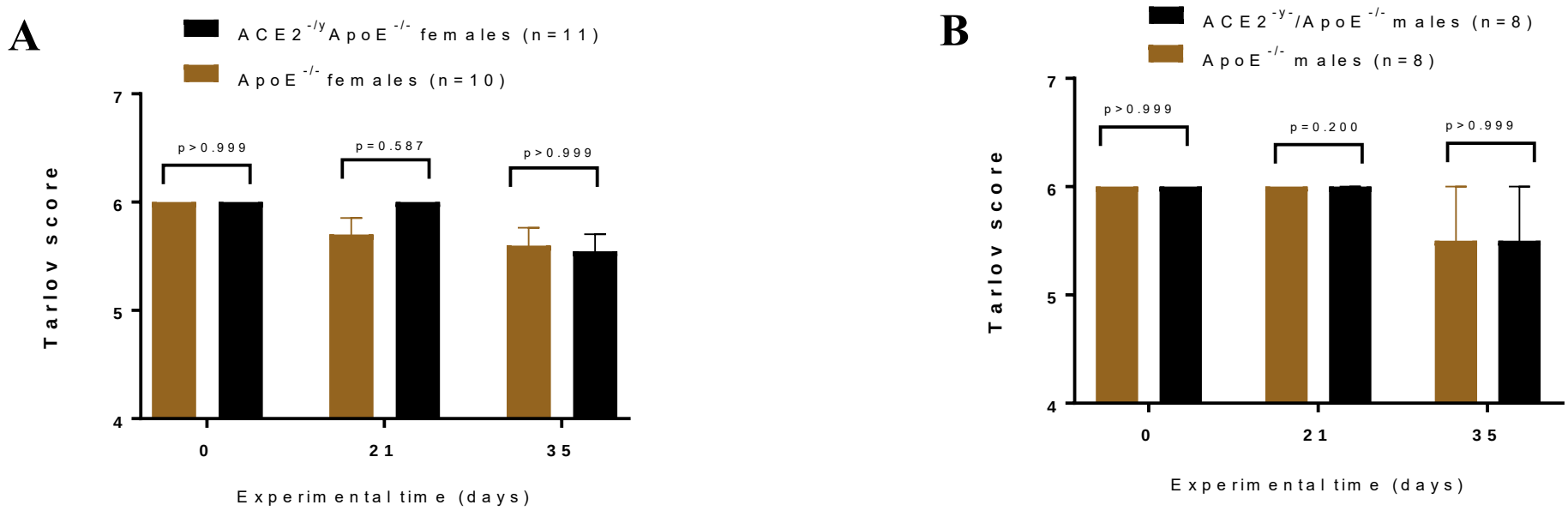


Figure R5.3. Effect of ACE2$^{-/y}$ on functional use score of the hindlimb (Tarlov) in the hindlimbs of mice with two stage HLI induction. A: Tarlov score of female *ACE2$^{-/y}$ApoE$^{-/-}$* vs female *ApoE$^{-/-}$* mice B: Tarlov score of male *ACE2^{-y-}ApoE$^{-/-}$* vs male *ApoE$^{-/-}$* mice. All results expressed are mean and SEM and all comparisons between groups were carried out by Mann Whitney U test.

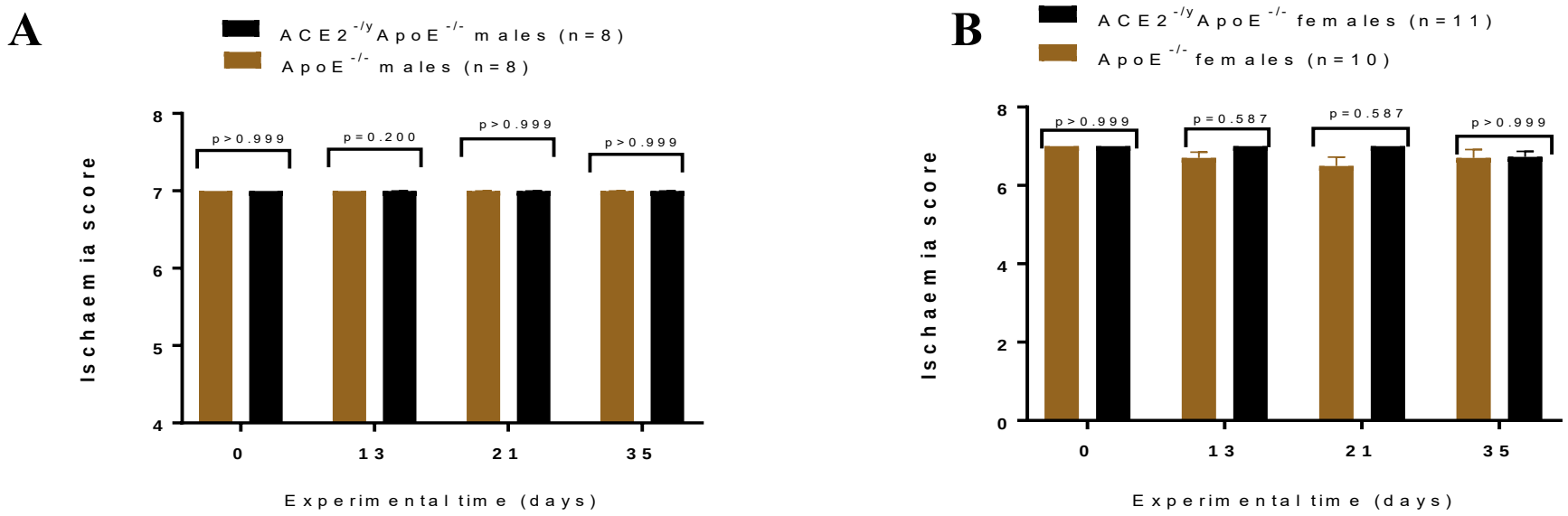


Figure R5.4 Effect of ACE2$^{-/y}$ on ischaemic score in the hindlimbs of mice with two stage HLI. A: Ischemia score of male *ACE2$^{-/y}$ApoE$^{-/-}$* vs male *ApoE$^{-/-}$* mice and B: Ischaemia score of female *ACE2$^{-/y}$ApoE$^{-/-}$* vs male *ApoE$^{-/-}$* mice. All results expressed are mean and SEM and all comparisons between groups were carried out by Mann Whitney U test.

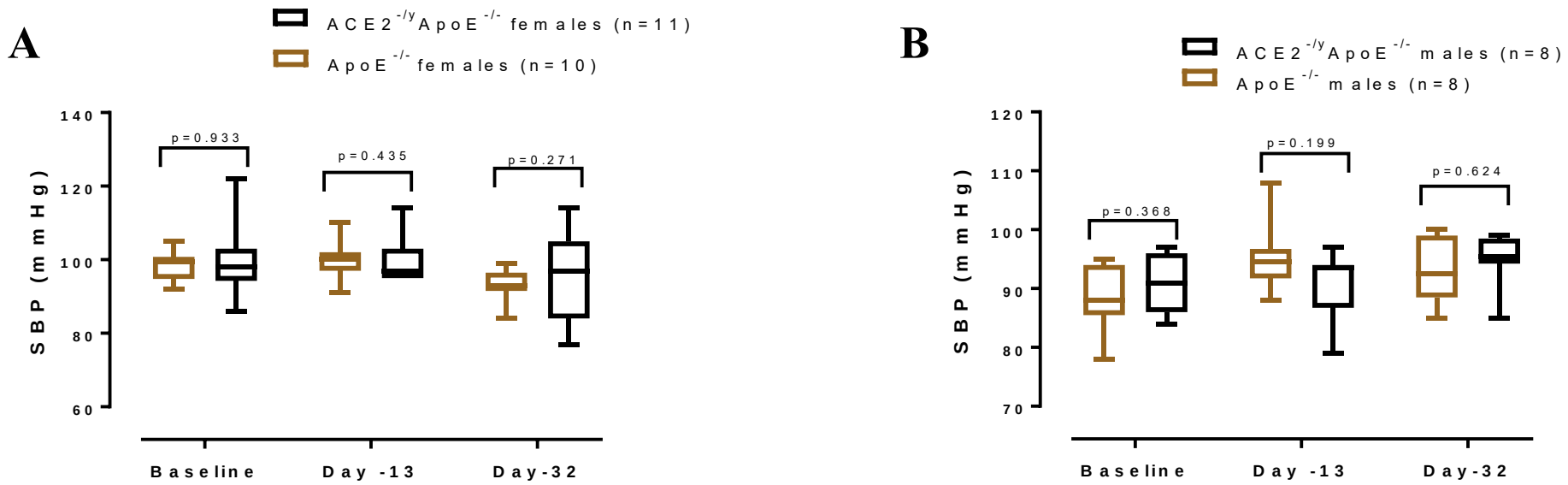


Figure R5.5: Effect of *ACE2*$^{-/y}$ in systolic blood pressure of *ApoE*$^{-/-}$ mice. A: SBP of female *ACE2*$^{-/y}$*ApoE*$^{-/-}$ vs male *ApoE*$^{-/-}$ mice. B: SBP of female *ACE2*$^{-/y}$*ApoE*$^{-/-}$ vs female *ApoE*$^{-/-}$ mice. Data expressed as median and interquartile ranges. Statistical comparisons between groups were carried out with a Mann-Whitney U test.

5.4 Discussion

The key finding of the study presented in this chapter was that *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice did not have greater severity of ischaemia and functional impairment compared to age and sex matched *ApoE*$^{-/-}$ control mice over 4 weeks after HLI induction. This is also the first study in which two-stage HLI was induced in female mice. The result showed that an ongoing ischaemia was present in the females and suggests that sex may not affect severity of ongoing ischaemia in the two-stage HLI model.

In the present study, *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice did not demonstrate an elevated SBP compared to age and sex matched *ApoE*$^{-/-}$ mice. The role of *ACE2*$^{-/y}$ in BP regulation in rodent studies is controversial with some studies showing increase in BP (Gurley, Allred et al. 2006, Thomas, Pickering et al. 2010, Rabelo, Todiras et al. 2016), with other studies demonstrating no differences compared to their respective control (Crackower, Sarao et al. 2002, Moritani, Iwai et al. 2013). In the present study adult mice were assessed compared to the relatively young mice used in previous studies (Silvestre, Bergaya et al. 2001). Mixed genetic backgrounds, high variability of BP data and a lack of statistical power has been suggested to result in these observations (Rabelo, Todiras et al. 2016). A protective role of the C57BL/6 background in BP regulation has also been suggested by previous studies (Oudit, Kassiri et al. 2009). The presence of compensatory mechanisms preventing higher Ang II levels in the *ACE2*$^{-/y}$ mice is also suggested by the lack SBP elevation. Ang II promotes e-NOS uncoupling for increased superoxide production and NO downregulation (Yamamoto, Ohishi et al. 2006, Alghamri, Weir et al. 2013, Moritani, Iwai et al. 2013). Lack of increase in Ang II levels in the *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice would not have promoted Ang II dependent decrease in NO bioavailability for greater ischaemia.

Lower Ang 1-7 levels leading to decreased phosphorylation of e-NOS subsequently lowering NO bioavailability in *ACE2*$^{-/y}$ was proposed to be one of the key mechanisms for greater ischaemia in *ACE2*$^{-/y}$ mice in the current study. Compensatory mechanisms may have prevented these effects to demonstrate comparable ischaemia in *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice and the *ApoE*$^{-/-}$ controls over the course of the experiment. Ang1-7 in circulation has a very short half-life (3-15 minutes) because of its physiological hydrolysation by ACE, neprilysin (NEP), and aminopeptidases (Yamada, Iyer et al. 1998, Breitling, Krauszman et al. 2015). In addition to being able to hydrolyse Ang1-7, NEP is able to generate Ang 1-7 and may have played a compensatory role in *ACE2*$^{-/y}$ mice. NEP is an endothelial cell surface zinc metallopeptidase

and kinetic studies of peptide cleavage has shown that NEP more efficiently hydrolyzed Ang I to Ang 1–7 compared with ACE2 (Rice, Thomas et al. 2004). Ang 1–7 is further cleaved by NEP to several smaller peptides (Stephenson and Kenny 1987, Rice, Thomas et al. 2004, Brar, Barrow et al. 2017). Mass spectrometry analysis has demonstrated that Ang 1-7 could be further cleaved by NEP to generate the dipeptide Ang 1–2 (Brar, Barrow et al. 2017). Very recently it has been reported that Ang 1-7 activity is critically dependent on its NEP mediated degradation to the bioactive Ang 1–2 dipeptide in relation to evoking an insulin secretory response (Brar, Barrow et al. 2017). It is interesting to explore whether modulation of NEP or Ang 1-2 may be beneficial for therapeutic revascularisation for limb ischaemia (Brar, Barrow et al. 2017).

Several potential metabolites are considered to play compensatory roles in *ACE2*$^{-/y}$ mice including vasoactive bradykinin (1-8), des-Arg-kallidin, dynorphin A, casomorphin, neurotensin 1 to 13, pyroglomulated-apelin 13 (pyr-apelin 13), apelin 17, and kinetensin (Donoghue, Hsieh et al. 2000, Thomas, Pickering et al. 2010, Wang, McKinnie et al. 2016). In relation to ischaemia, apelins (pyr-apelin 13 and apelin 17) may have had important compensatory roles preventing greater ischaemia in the *ACE2*$^{-/y}$ mice. Pyr-apelin 13 and apelin 17 are the dominant apelin peptides found *in vivo*. ACE2 metabolises and partially inactivates pyr-apelin and apelin 17 (Wang, McKinnie et al. 2016). The half-lives of apelins are extremely short and this has been reported to be prolonged in *ACE2*$^{-/y}$ plasma (Wang, McKinnie et al. 2016). interesting compensatory peptides highly likely preventing greater ischaemia in the *ACE2*$^{-/y}$ mice. Pyr-apelin 13 and apelin 17 has been shown to stimulate a robust increase in NO production concordant with increased phosphorylation of Akt and e-NOS in human umbilical vascular endothelial cells (Donoghue, Hsieh et al. 2000, Wang, McKinnie et al. 2016). Studies on apelin in the acute model of HLI have demonstrated that apelin deficiency reduces the rate of blood flow recovery and worsens necrosis (Kidoya, Naito et al. 2010, Andersen, Hilberg et al. 2011). Furthermore, apelin administration was able to increase the number of larger vessels and decrease necrosis in a model of HLI in mice (Kidoya, Naito et al. 2010). Apelins have been described to stimulate functional and morphological maturation of neo-vessels after cell transplantation in ischaemic tissues. Immature vessel formation is a major problem that hinders use of effective therapeutic angiogenesis agents such as VEGF and stem cells. Apelin is an agent that has been seen as a promising combinatorial peptide for therapeutic angiogenesis with stem cells and gene therapy (Kidoya, Naito et al. 2010, Samura, Morikage et al. 2016). It would be interesting to

investigate whether newly developed ACE2 resistant apelin analogues have potential for therapeutic angiogenesis in limb ischaemia.

In a recent study, it has been reported that resveratrol upregulates ACE2 expression within the aorta of a mouse model (Moran, Biros et al. 2017). Furthermore, resveratrol has also been reported to upregulate adipose expression of ACE2 in hyperlipidaemic mice (Oliveira Andrade, Paraiso et al. 2014). Interestingly, a recent randomised trial in patients with intermittent claudication due to PAD reported that resveratrol does not improve maximum walking distance (McDermott, Leeuwenburgh et al. 2017). These findings suggest that ACE2 upregulation may not have any therapeutic value for PAD in line with the findings from the current study.

Strengths of this study include the use of a novel mouse model with persistent HLI, investigation of *ACE2*$^{-/y}$ in both sex and the use of adult mice on an atherosclerosis (*ApoE*$^{-/-}$) background. Further work to address weakness of this study include the measurement of Ang II levels or activity, measurement of circulating NO and Ang 1-7. Future work may also be focused on measuring levels or activity of ACE2 metabolites or compensatory proteins such as apelins, NEP and Ang 1-2 in *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice. Additional limitations include the lack of a chronic model of slow onset of ischaemia in which slow onset of ischaemia takes longer than two weeks. Further evidence is needed to understand the role of ACE2 in modulating shear stress so that a clear conclusion may be determined regarding the role of ACE2 in PAD.

In conclusion this study demonstrates that *ACE2*$^{-/y}$*ApoE*$^{-/-}$ mice do not have increased severity of HLI compared to *ApoE*$^{-/-}$ controls 4 weeks after two-stage HLI induction. The findings suggest that ACE2 may not play an important role in improving blood supply in an experimental model HLI. While ACE2 upregulation remains an exciting strategy for a variety of diseases, the results of this study from the two stage mouse model of HLI does not encourage the pursuit of this strategy for the pharmacological management of PAD in clinical trials and ACE2 is unlikely to be an important target for improving limb blood supply or function in patients with PAD.

Chapter 6
Concluding discussions and future directions

6.1 Discussion: Development of a two-stage mouse model of lower limb ischaemia

Treatments for limb ischaemia in PAD is an unmet medical need (Vemulapalli, Dolor et al. 2015). Advancements in treatments have been limited by the inability of currently used pre-clinical animal models and outcome measures to successfully predict clinical effectiveness (Lederman, Mendelsohn et al. 2002, Rajagopalan, Mohler et al. 2003, Olea, Vera Janavel et al. 2009, Belch, Hiatt et al. 2011, Annex 2013, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Moxon et al. 2015, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016, McDermott, Ferrucci et al. 2017, Rigato, Monami et al. 2017). This is likely attributable to pre-clinical study characteristics including the reliability of animal model used, study design and endpoints assessed (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016). Recommendations for a mouse model of HLI to be clinically relevant in order to better translate findings include: simulating the pathophysiology of PAD through slow onset of severe ischaemia and functional impairment; incorporating more representative pathology, such as concurrent atherosclerosis; incorporating common risk factors present in patients, such as older age, diabetes or smoking; using outcome assessments common in human trials such as treadmill testing; and demonstrating clinically relevant response to established treatments of PAD such as exercise training (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016).

A major disadvantage of currently used pre-clinical animal models to simulate PAD is the rapid natural recovery of blood supply to ischaemic tissues which occurs after HLI induction (Aranguren, Verfaillie et al. 2009, Lotfi, Patel et al. 2013, Mohamed Omer, Krishna et al. 2016). Evidence suggests that in the most commonly used mouse model of HLI which is developed through the acute ligation and excision of the FA (Dragneva, Korpisalo et al. 2013, Krishna, Omer et al. 2016) sudden changes in fluid shear stress within collateral arteries promote arteriogenesis and angiogenesis to restore blood supply to the ischaemic tissues (Topper and Gimbrone Jr 1999, Garcia-Cardena, Comander et al. 2001, Tang, Chang et al. 2005). Ameroid constrictors placed at the ends of the FA have been suggested to modulate rapid shear stress changes in previous studies (Tang, Chang et al. 2005, Yang, Tang et al. 2008). However, only mild ischaemia is achieved by ameroid constriction (Tang, Chang et al. 2005). This suggests that ameroid constriction alone may be inadequate to overcome efficient collateral compensation of blood supply. In order to develop the slow onset of severe ischaemia, excising the FA after slow onset of mild ischaemia by the ameroids may mitigate blood supply through collaterals arising from the FA.

In the first experimental work of this thesis (chapter 1, study 1) a new mouse model of HLI was developed through a two-stage method which involved slow FA constriction using ameroids at the ends of the FA for 2 weeks, followed by excision of the FA to achieve total occlusion and severe ischaemia. Assessment of limb blood supply in the two-stage model by LDI showed that ameroid placement alone did not achieve significant ischaemia after 2 weeks. The second surgery of excision of the FA resulted in significantly severe ischaemia than the ameroids alone. In comparison to the acute model of HLI, the two-stage model of HLI developed in this thesis showed minor blood flow recovery immediately following ischaemia induction and after 1 week from HLI induction the severity of HLI persisted. In line with the finding of severe prolonged ischaemia assessed by LDI in the new mouse model, clinical signs manifested as a result of ischaemia induction were greater in the two-stage HLI model as assessed by clinical scoring. Together with the main technique of ischaemia induction, the new experimental model which was developed incorporated additional characteristics of PAD patients including atherosclerosis (through *ApoE*$^{-/-}$) and old age. Atherosclerosis is the leading cause of PAD and atherosclerosis simulating mouse models such as *ApoE*$^{-/-}$ mice develop significant atherosclerotic lesions spontaneously and exhibit delayed recovery from ischaemia (Kang, Albadawi et al. 2008, Dragneva, Korpisalo et al. 2013, Lotfi, Patel et al. 2013, Krishna, Omer et al. 2016, Mohamed Omer, Krishna et al. 2016).

In the main study of chapter 3 in this thesis, greater functional impairment was observed in the two-stage HLI model than the acute HLI model through the clinical scoring method. It is not common for pre-clinical studies to quantitatively assess functional impairment in animal models of HLI and functional assessments mirroring the clinical trial assessments are lacking in pre-clinical studies of HLI. Using outcome assessments common in human trials such as treadmill testing is a suggested recommendation for improving translatability of pre-clinical findings (Krishna, Omer et al. 2016). Treadmill exercise testing is the most commonly used objective outcome measure in randomised clinical trials studying interventions to improve walking performance in people with PAD.. In the current study assessment of treadmill performance demonstrated the two-stage model of HLI had greater treadmill walking impairment than the acute model of HLI.

The demonstration of functional impairment and similar responses to known interventions in humans are important characteristics of an ideal mouse model for PAD (Krishna, Omer et al. 2016). Current guidelines and a considerable body of evidence support the clinical benefits of a supervised exercise program in improving pain free walking performance, physical capacity

and quality of life in PAD patients (Norgren, Hiatt et al., Norgren, Hiatt et al. 2007, European Stroke, Tendera et al. 2011, Brass 2013, Hiatt, Armstrong et al. 2015, Gerhard-Herman, Gornik et al. 2016, Golledge, Singh et al. 2019). The mechanisms associated with improvements in limb symptoms as a result of exercise intervention in patients are not clearly understood. The majority of studies in patients have consistently shown a poor correlation between leg blood flow and walking ability with no increase in blood flow assessed by ABPI following an exercise programme, even when significant improvements in walking ability have been reported (Stewart , Hiatt et al. 2002, Askew, Green et al. 2005, Parmenter, Raymond et al. 2010, Duscha, Robbins et al. 2011, Meneses, Nam et al. 2018). However, in the experimental model of acute HLI, exercise training augments recovery of blood supply to ischaemic muscles (Yang, Dinn et al. 1990, Yang, Prior et al. 2008, Cheng, Kuzuya et al. 2010, Rokutanda, Izumiya et al. 2011).

Exercise intervention after ischaemia induction in the two-stage HLI model promoted treadmill walking distance independent of a change in limb blood supply. This is a finding similar to that described in PAD patients (Watson, Ellis et al. 2008, Parmenter, Raymond et al. 2010, Malgor, Alahdab et al. 2015) and validates the two-stage HLI model for assessing interventions for improving functional impairment.

6.2 Future directions: Part 1

A number of potential future directions have arisen from the findings of the studies in the first Chapter. The main finding showed that the new two-stage model of HLI had severe ongoing ischaemia. Suggested future studies for this finding includes characterisation of mechanisms associated with ongoing severe ischaemia. This includes histological characterisation of the micro-vasculature to determine changes in arteriogenesis and angiogenesis and molecular characterisation of shear stress modulators such as the mechano-transducer transient receptor potential vanilloid 4.

The primary study in this chapter utilised older *ApoE*$^{-/-}$ mice to better simulate the characteristics of PAD patients. It is known however, that other co-morbidities such as diabetes affects blood flow recovery to limb ischaemia in patients. DM is a strong risk factor for PAD (Fowkes, Rudan et al. 2013) and patients with DM-associated PAD are more likely to progress to CLI (Thiruvoipati, Kielhorn et al. 2015). The amputation rate for patients with DM is five

times higher than those without DM and PAD in the presence of DM is a leading current clinical problem (Mellière, Berrahal et al. 1999). Establishing a mouse model of two-stage HLI in the presence of DM may aid in developing effective treatments for translation for PAD patients with DM. In addition, angiogenic capability of some other mouse strains (e.g. BALB/c) is lower than that of C57BL/6 employed in this thesis (Scholz, Ziegelhoeffer et al. 2002, Fukino, Sata et al. 2003, Helisch, Wagner et al. 2006, Chalothorn, Clayton et al. 2007, Thomas, Thirumaran et al. 2016). It is possible that the surgical procedures proposed in this study may produce greater severity of ongoing ischaemia in BALB/c mice and future research investigating this is warranted.

The new two-stage slow onset HLI mouse model demonstrated a clinically relevant response to exercise training in that exercise training improved treadmill walking distance independent to changes in limb blood supply assessed by LDI. Future mechanistic studies are needed to characterise the underlying cellular and molecular mechanisms related to improvements in limb function independent of changes to limb blood supply. Suggestions include characterising ischaemic muscle metabolism, oxidative stress, inflammation, mitochondrial function, endothelial function and the microvasculature.

6.3 Part 2 Discussion: The effect of metformin in two-stage HLI

Repurposing existing treatments for modulating e-NOS to improve revascularisation in limb ischaemia may be a suitable strategy for recognising effective treatments urgently needed for PAD patients. Evidence suggests the drug metformin may have potential to be repurposed to improve blood supply to limb ischaemia (Sirtori, Franceschini et al. 1984, Montanari, Bondioli et al. 1992) by modulating e-NOS activity through AMPKα activity (Takahashi, Shibata et al. 2015). Additional suggested mechanisms through which metformin may modulate improvement in blood supply include AMPKα dependent attenuation of oxidative stress by downregulating expression of TXNIP and improving mitochondrial biogenesis via PGC1α upregulation (Jager, Handschin et al. 2007, Fernandez-Marcos and Auwerx 2011, Chong, Chan et al. 2014, Zhang, Pang et al. 2015).

In the second experimental chapter presented in this thesis (Chapter 4), the biguanide metformin was investigated for improving blood supply in the newly developed two-stage model of HLI. Metformin administration promoted recovery of hindlimb blood supply in the novel mouse model of ongoing ischemia. AMPKα activity was upregulated in mice receiving

metformin suggested by the increased protein expression of phospho-AMPKα to total-AMPKα, suggesting that recovery of blood supply from an established steady state was improved via AMPKα dependent mechanisms. Metformin intervention resulted in upregulation of AMPKα activity and e-NOS activity with increased circulating NO levels. Additionally decreased gene and protein expression of oxidative stress marker TXNIP and increased gene and protein expression of the mitochondrial biogenesis marker PGC1α were indicated in the ischaemic tissues of mice receiving metformin.

In this study, the effect of metformin intervention on treadmill walking performance was also examined. Unlike the improvement in blood supply, metformin intervention did not promote hindlimb function assessed by treadmill walking distance in the two-stage model of HLI within the experimental period. A limitation of this study for determining the treadmill walking performance in the mice was the sample size. Sample size for this study was estimated for the outcome measure of LDI. This study was limited in sample size to derive a clear conclusion of the effect of metformin intervention on treadmill walking capacity in the two-stage experimental model of HLI.

Additional possible limitations could be the length of time on intervention to derive functional benefits and the dose used. The effect of metformin was examined for only 4 weeks in mice with two-stage HLI at the currently prescribed daily human equivalent dosage of 2.0g/80kg person. Length of time for improvement to be apparent is a feature of current management strategies for PAD such as with supervised walking exercise interventions which typically require months before substantial improvement in walking performance is realised (McDermott 2013). The dose used in this study was the currently prescribed human dose for managing hyperglycaemia in diabetes patients. In this study the improvement in blood supply by metformin was only observed 2 weeks after commencing the intervention. This suggests that examining a higher dose at the start of intervention may be relevant for faster improvement of blood supply by metformin intervention.

6.4 Future directions: Part 2

The findings of this chapter presents opportunities to undertake a number of future studies. Future studies are required with larger sample sizes and longer duration to determine the effect

of metformin on treadmill walking performance. Further investigations are also needed with different doses to determine the best dose for optimum efficacy of metformin for improving revascularisation and limb function.

In this study the underlying changes were characterised to be associated with the metabolic mechanisms for improving blood supply. Future studies are recommended to gather histological evidence of revascularisation and studies need to explore additional mechanisms. An example of potential mechanisms is suggested by a recent report on AMPKα1 related mechanism involving the regulation of the amount and function of monocytes and macrophages in ischaemic tissues (Zhu, Zhang et al. 2016). It has been shown that deletion of AMPKα1 decreases the accumulation of macrophages in the muscle from ischaemic hind limbs *in vivo* and impairs arteriogenesis by regulating the amount and function of monocytes and macrophages in ischaemic tissues (Zhu, Zhang et al. 2016) Macrophage AMPKα1 plays an essential role in arteriogenesis through its regulation of growth factor generation (Zhu, Zhang et al. 2016). AMPKα is one of the major signalling pathways which govern macrophage behaviour which exhibit marked phenotype heterogeneity. Phenotypically polarised macrophages are generally termed pro-inflammatory M1 and anti-inflammatory M2 (Parisi, Gini et al. 2018). In addition, based on expression levels of Ly6C, an inflammatory monocyte marker, monocytes subsets are grouped as $Ly6C^+$ monocytes and $Ly6C^-$ monocytes (Thomas, Tacke et al. 2015). During vascular inflammation, $Ly6C^-$ monocytes are recruited to tissues and differentiate into M2 macrophages, which secrete anti-inflammatory cytokines and contribute to tissue repair (Wynn and Vannella 2016). Accordingly, how metformin can modulate the differentiation of $Ly6C^-$ monocytes into M2 macrophages remains the subject of ongoing interesting studies. Upregulation of AMPKα1 by metformin in $Ly6C^-$ monocytes may enhance their recruitment to ischaemic tissues and facilitate terminal differentiation into M2 macrophages which may promote vascular regeneration and tissue repair. The modulation of immune response on revascularisation of limb ischaemia, specifically macrophage modulation of revascularisation by metformin or AMPK activators would be interesting future studies.

Inhibition of complex 1 of the mitochondrial respiratory chain resulting in AMPKα activation causing downstream signals is reported to be the main mechanism of action of metformin in order to have its effects. A recently described AMPK activator, R419, activates AMPK by inhibiting the mitochondrial complex 1 similar to metformin (Marcinko, Bujak et al. 2015). R419, has been shown to improve treadmill running capacity by over 30% in obese mice fed a

high-fat diet (Marcinko, Bujak et al. 2015). Metformin's ability to inhibit complex 1 has been reported to be mild and transient (Foretz, Guigas et al. 2014). Therefore, a strong inhibitor of complex 1 resulting in AMPK activation may generate more favourable improvements in blood supply and functional outcomes in HLI. Future studies investigation novel AMPK activators are recommended.

In addition, the model in which metformin was tested lacked DM. Although hyperglycaemia in PAD patients with DM is likely to be managed with metformin, the development of a two-stage mouse model of HLI with DM may offer potential to characterise beneficial mechanisms of metformin in the pathophysiology of PAD in the presence of DM. Furthermore, future studies with AMPK activators in the two-stage mouse model of HLI with DM may offer new translational therapeutic opportunities for PAD patients with DM.

6.5 Part 3 Discussion: The effect of Angiotensin converting enzyme 2 deficiency in experimental limb ischaemia

Endogenous compounds which modulate e-NOS activity have been suggested to play important roles in recovery of blood supply to limb ischaemia (Forte, Conti et al. 2016). Evidence suggests ACE2 could be an important modulator of e-NOS activity (Zhang, Wang et al. 2014) by promoting e-NOS phosphorylation through Ang 1-7 synthesis and limiting e-NOS uncoupling by AngII degradation.

In the last experimental chapter of this thesis, $ACE2^{-/y}$ was investigated for its role in limb ischaemia employing the two-stage mouse model of HLI. $ACE2^{-/y}ApoE^{-/-}$ mice did not have greater severity of ischaemia compared to age and sex matched $ApoE^{-/-}$ control mice over 4 weeks after HLI induction. The findings also showed that, $ACE2^{-/y}ApoE^{-/-}$ mice did not demonstrate an elevated BP compared to age and sex matched $ApoE^{-/-}$ mice. This suggests the presence of compensatory mechanisms in the $ACE2^{-/y}ApoE^{-/-}$ mice (Crackower, Sarao et al. 2002, Gurley, Allred et al. 2006, Thomas, Pickering et al. 2010, Moritani, Iwai et al. 2013, Rabelo, Todiras et al. 2016). The presence of compensatory mechanisms also suggests the possibility that NO and e-NOS activity may not have been significantly affected by $ACE2^{-/y}$ to severely impair blood flow in the two-stage HLI model. Suggestions of compensatory mechanisms in $ACE2^{-/y}$ mice which may have prevented an important role of ACE2 in limb ischaemia include Ang 1-7 and Ang 1-2 generation by NEP and increased apelin signaling (Stephenson and Kenny 1987, Rice, Thomas et al. 2004, Kidoya, Naito et al. 2010, Andersen, Hilberg et al. 2011, Brar, Barrow et al. 2017).

6.6 Future directions: Part 3

Future studies suggested for the findings from this thesis chapter includes the measurement of NO levels between *ACE2*$^{-/y}$ and *ApoE*$^{-/-}$ controls. Furthermore, apelins could be investigated for a role in limb ischaemia employing the two-stage mouse model of HLI.

6.7 Thesis conclusions

The main conclusion of this thesis are:

1) The novel two-stage mouse model of HLI demonstrated severe ongoing HLI and functional impairment. Exercise training augmented treadmill walking distance in the new mouse model which was independent of changes to limb blood supply and mirrors patient response to exercise therapy.

2) Metformin improved limb blood supply in the two-stage mouse model of ongoing HLI. Improvement in blood supply was associated with the upregulation of AMPKα activity, increased activation of e-NOS in the ischaemic muscles, increased bioavailability of circulating NO, increased expression of PGC1α and reduced expression of TXNIP. The study suggests metformin may have potential in improving limb blood supply in PAD patients.

3) *ACE2*$^{-/}$ did not worsen limb blood supply after two-stage HLI induction in the new mouse model suggesting ACE2 may not play an important role in limb ischemia. The results of this study from the two stage mouse model does not encourage the pursuit of ACE2 for pharmacological management of PAD in clinical trials and suggests ACE2 is unlikely to be an important target for improving limb blood supply or function in patients with PAD. However, further evidence is needed with improved mouse models of HLI to confirm the role of ACE2 in PAD.

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Appendix

A1. Ethics Approvals

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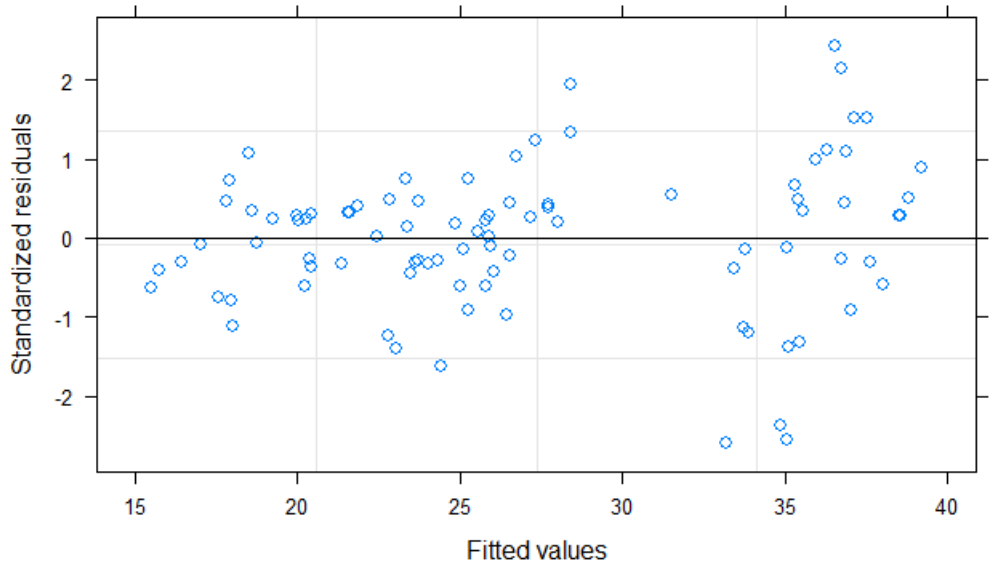
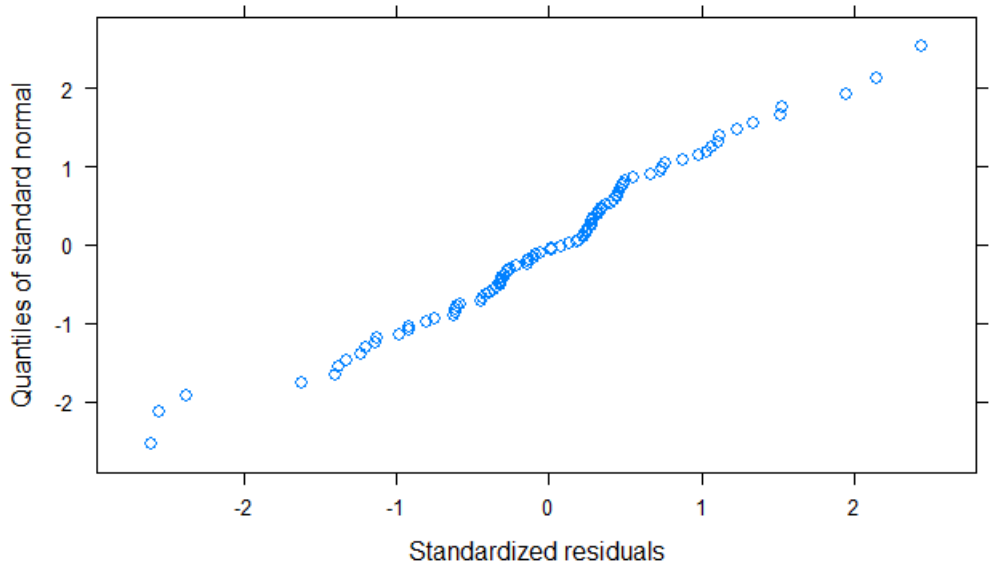
A2: Linear mixed effects results

A2.1 LME model analysis comparing treadmill data between exercise intervention and control groups

```
Linear mixed-effects model fit by REML
 Data: Exercise.treadmill.data
       AIC      BIC    logLik
  600.0009 619.4474 -292.0004

Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept) Residual
StdDev:     2.75153 6.626358

Fixed effects:   Sqrt.Treadmill.dist ~ Treatment * TIME
                    Value  Std.Error DF  t-value p-value
(Intercept)       18.135090  1.852557 56 9.789219  0.0000
Treatment1         3.541022   2.619912 28 1.351581  0.1873
TIME5              7.292634   2.419604 56 3.013978  0.0039
TIME7              7.975062   2.419604 56 3.296020  0.0017
Exercise:TIME5     6.184013   3.421837 56 1.807220  0.0761
Exercise:TIME7     7.183232   3.421837 56 2.099233  0.0403
Exercise:TIME5     6.184013   3.421837 56 1.807220  0.0761
Exercise:TIME7     7.183232   3.421837 56 2.099233  0.0403
 Correlation:
                  (Intr) Trtmn1 TIME5  TIME7  T1:TIME5
Exercise          -0.707
TIME5             -0.653  0.462        Correlation:
                  (Intr) Trtmn1 TIME5  TIME7  T1:TIME5
Exercise          -0.707
TIME5             -0.653  0.462
TIME7             -0.653  0.462  0.500
Exercise:TIME5     0.462 -0.653 -0.707 -0.354
Exercise:TIME7     0.462 -0.653 -0.354 -0.707  0.500

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max

TIME7             -0.653  0.462  0.500
Exercise:TIME5     0.462 -0.653 -0.707 -0.354
Exercise:TIME7  0.462 -0.653 -0.354 -0.707  0.500

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.6046838 -0.4259500  0.1060574  0.4542599  2.4401130

Number of Observations: 90
Number of Groups: 30
```

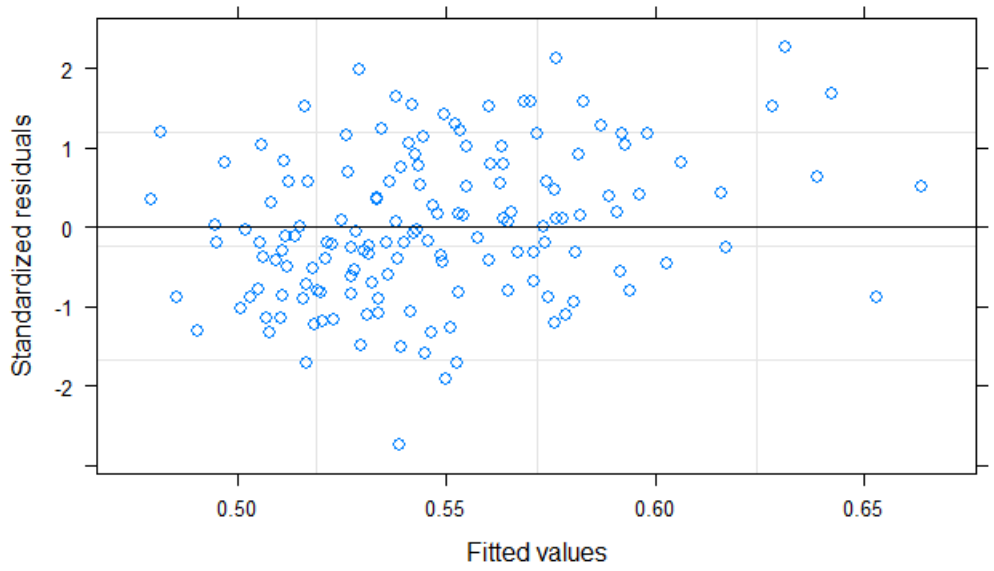


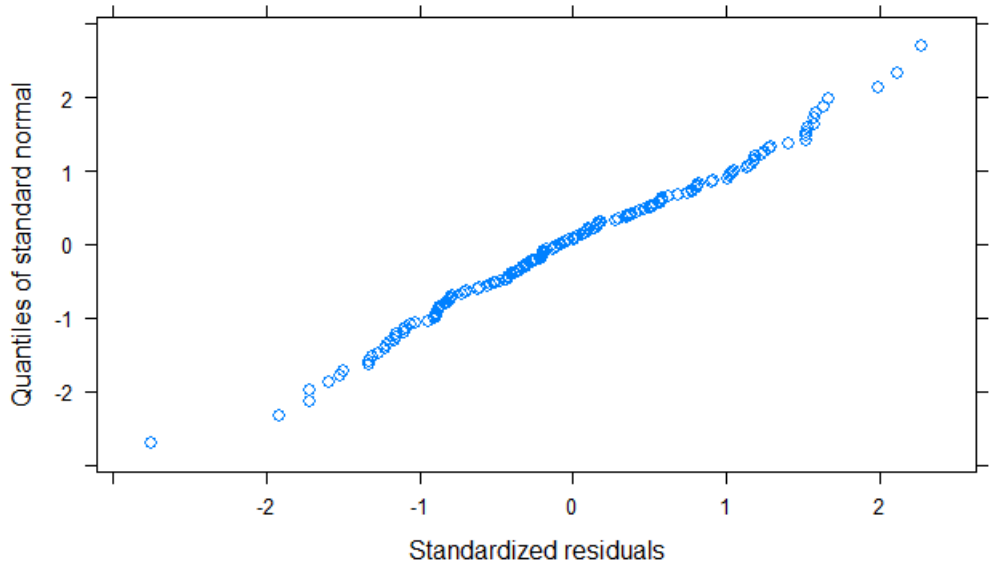


A2.2 LME model analysis comparing LDI data between exercise intervention and control groups

```
Linear mixed-effects model fit by REML
 Data: Exercise.LDI.data
        AIC       BIC   logLik
  -216.6022 -198.7005 114.3011

Random effects:
 Formula: ~1 | Mouse.ID
```

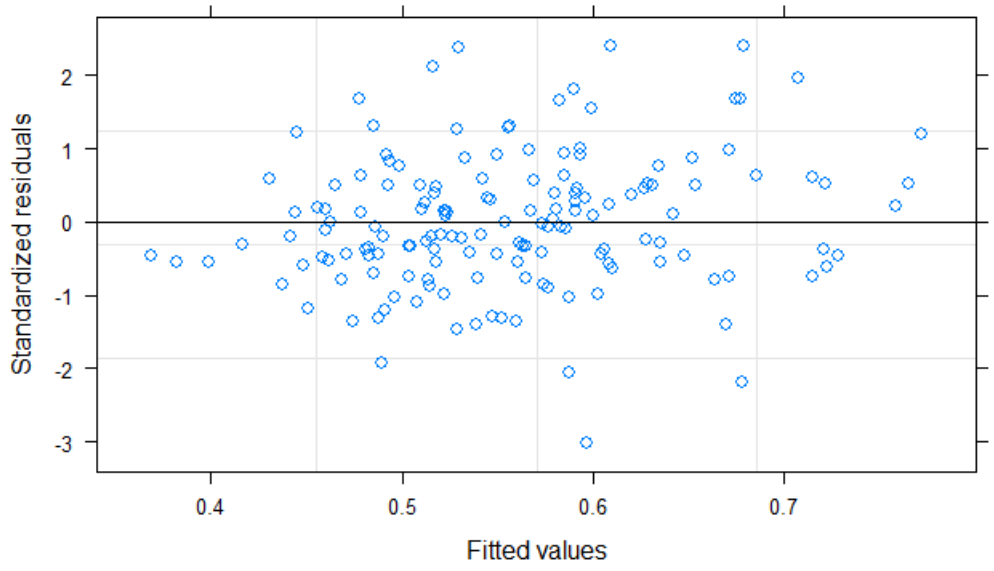
```
        (Intercept)   Residual
StdDev:   0.0455824Linear mixed-effects model fit by REML
 Data: Exercise.LDI.data
        AIC       BIC   logLik
  -216.6022 -198.7005 114.3011

Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept)   Residual
StdDev:   0.0455824 0.09362099

Fixed effects: LDI.ratio ~ Treatment * Time
                     Value  Std.Error  DF   t-value p-value
(Intercept)      0.5318895 0.03620481 118 14.691128  0.0000
Treatment2      -0.0295530 0.05120133  28 -0.577192  0.5684
Time             0.0003047 0.00100269 118  0.303871  0.7618
Exercise:Time    0.0012437 0.00141801 118  0.877043  0.3822
 Correlation:
                (Intr) Trtmn2 Time
Treatment2      -0.707                0.303871  0.7618
Treatment2:Time  0.0012437 0.00141801 118  0.877043  0.3822
 Correlation:
                (Intr) Trtmn2 Time
Exercise        -0.707
Time            -0.897  0.634
Exercise:Time    0.634 -0.897 -0.707

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.75349317 -0.71719604 -0.08217634  0.67238675  2.27405272

Number of Observations: 150
Number of Groups: 30
```

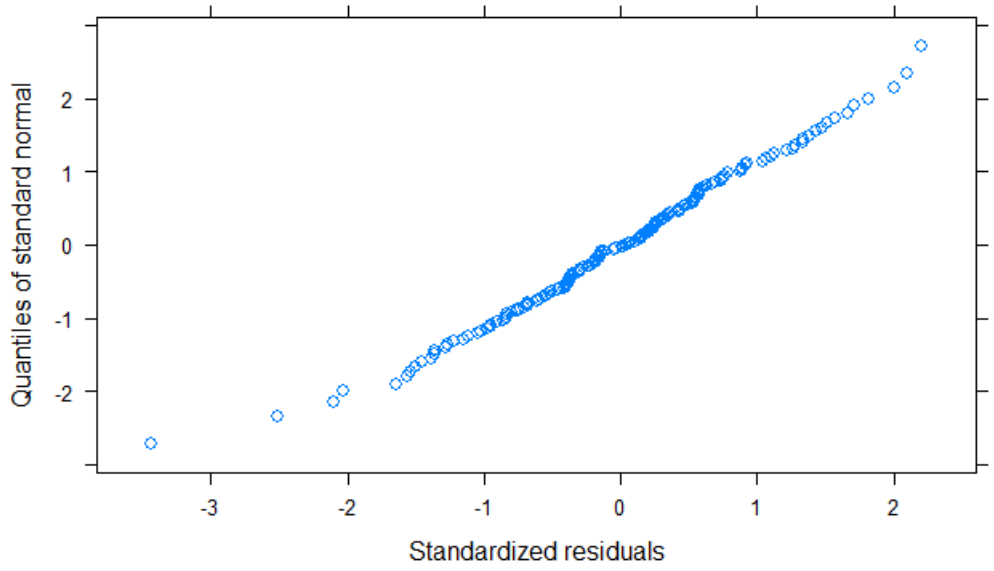




A2.3 LME model analysis comparing LDI data between metformin administered and vehicle administered groups

```
Linear mixed-effects model fit by REML
 Data: Metform.LDI.data
        AIC      BIC    logLik
  -221.8056 -203.702 116.9028

Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept)  Residual
StdDev:  0.06836679 0.0895167

Fixed effects: LDI.ratio ~ Treatment * Time
                     Value  Std.Error  DF   t-value p-value
(Intercept)      0.3834909 0.03601226 122 10.648898  0.0000
Treatment1       0.1306407 0.05177089  29  2.523439  0.0174
Time             0.0061892 0.00092828 122  6.667362  0.0000
Treatment1:Time -0.0057637 0.00133449 122 -4.318995  0.0000
 Correlation:
                (Intr) Trtmn1 Time
Treatment1      -0.696
Time            -0.835  0.581
Treatment1:Time  0.581 -0.835 -0.696

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.03007014 -0.54552927 -0.06467682  0.51112205  2.41198193

Number of Observations: 155
Number of Groups: 31
```

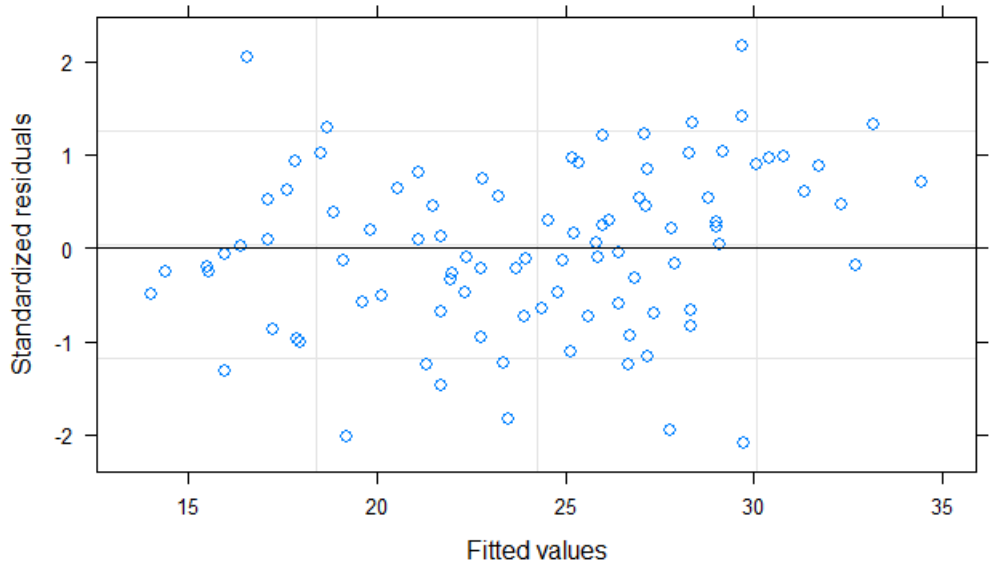


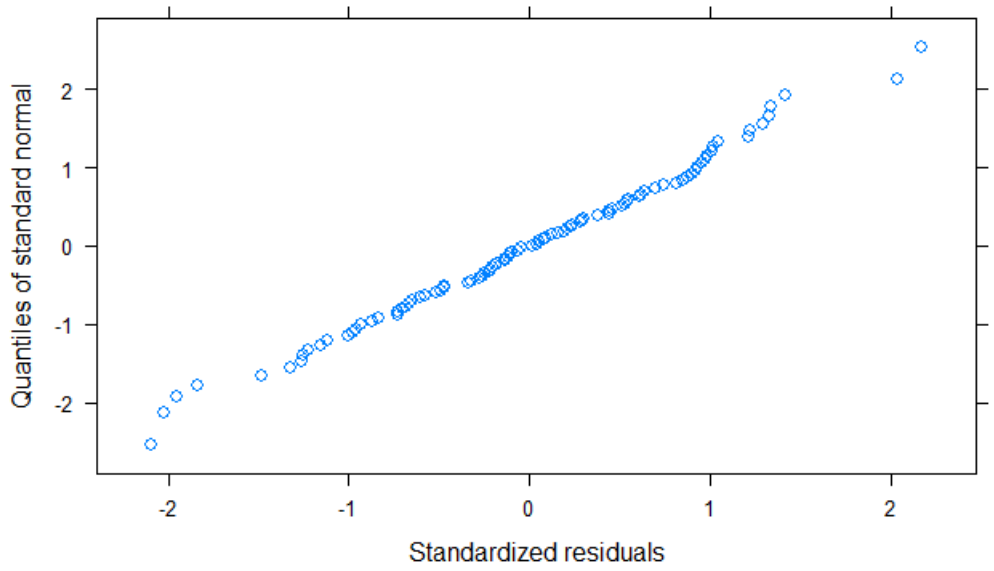


A3.4 LME model analysis comparing treadmill walking distance data between metformin administered and vehicle administered groups

```
Linear mixed-effects model fit by REML
 Data: Metform.treadmill.data
       AIC      BIC    logLik
  1275.699 1295.427 -629.8497

Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept) Residual
```

```
StdDev:    166.1586 270.4254

Fixed effects: Treadmill.distance ~ Treatment * TIME
                    Value Std.Error DF   t-value p-value
(Intercept)     347.4133  81.95063 58  4.239300  0.0001
Treatment        43.0804 114.07045 29  0.377665  0.7084
TIME5           333.0200  98.74541 58  3.372511  0.0013
TIME7           382.1733  98.74541 58  3.870290  0.0003
Treatment:TIME5 -31.4512 137.44779 58 -0.228823  0.8198
Treatment:TIME7 185.6829 137.44779 58  1.350934  0.1820
 Correlation:
                (Intr) Trtmnt TIME5  TIME7  T:TIME5
Treatment       -0.718
TIME5           -0.602  0.433
TIME7           -0.602  0.433  0.500
Treatment:TIME5  0.433 -0.602 -0.718 -0.359
Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept) Residual
StdDev:    3.287717 5.333549

Fixed effects: Sqrt.Treadmill.dist ~ Treatment * TIME
                     Value Std.Error DF   t-value p-value
(Intercept)     18.135090  1.617732 58 11.210198  0.0000
Treatment        0.757278  2.251787 29  0.336301  0.7391
TIME5            7.292634  1.947537 58  3.744542  0.0004
TIME7            7.975062  1.947537 58  4.094948  0.0001
Treatment:TIME5 -0.499741  2.710856 58 -0.184348  0.8544
Treatment:TIME7  3.212063  2.710856 58  1.184889  0.2409
 Correlation:
                (Intr) Trtmnt TIME5  TIME7  T:TIME5
Treatment       -0.718
TIME5           -0.602  0.432
TIME7           -0.602  0.432  0.500
Treatment:TIME5  0.432 -0.602 -0.718 -0.359
Treatment:TIME7  0.432 -0.602 -0.359 -0.718  0.500

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.0948786 -0.6015180  0.0147499  0.6206117  2.1711432

Number of Observations: 93
Number of Groups: 31
```

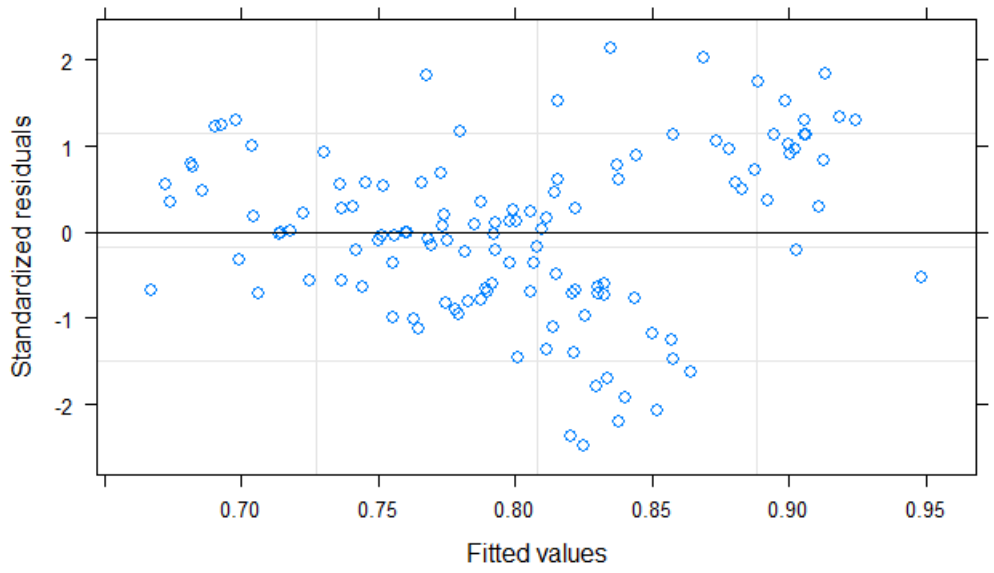


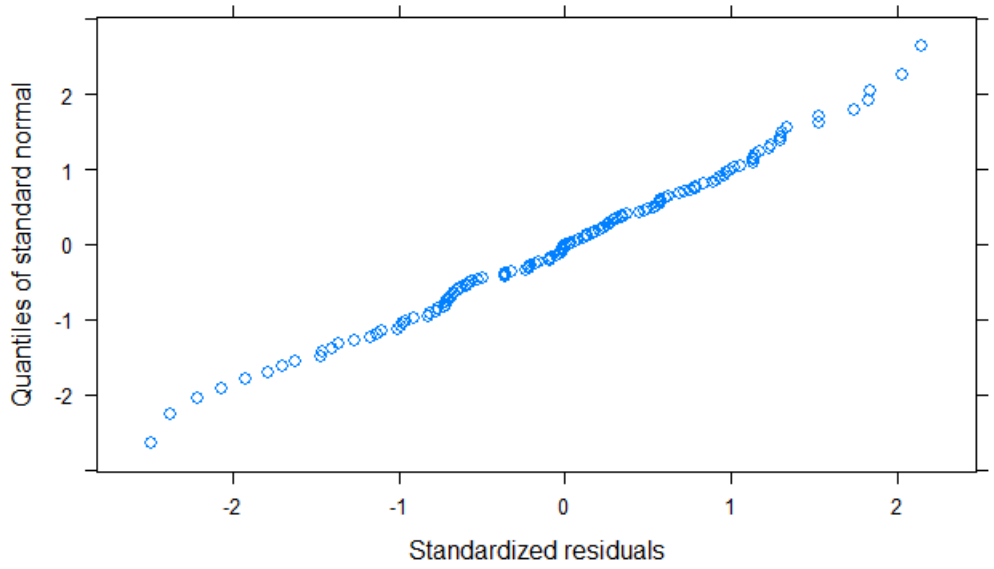


A2.5 LME model analysis comparing LDI data between female *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ groups.

```
Linear mixed-effects model fit by REML
 Data: ACE2fem.LDI.data
        AIC      BIC    logLik
  -108.5965 -91.7724 60.29826

Random effects:
 Formula: ~1 | Mouse.ID
        (Intercept)  Residual
StdDev:  0.03371165 0.1286595
```

```
Fixed effects: Sqrt.LDI.ratio ~ Group * Timepoint
                     Value  Std.Error  DF   t-value p-value
(Intercept)      0.9051229 0.03488354 103 25.946989  0.0000
Group           -0.0085216 0.04819854  19 -0.176802  0.8615
Timepoint       -0.0053759 0.00123271 103 -4.361041  0.0000
Group:Timepoint  0.0021373 0.00170324 103  1.254856  0.2124
 Correlation:
                (Intr) Group  Timpnt
Group           -0.724
Timepoint       -0.825  0.597
Group:Timepoint  0.597 -0.825 -0.724

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.48823366 -0.67523694  0.01713875  0.67139896  2.14353107

Number of Observations: 126
Number of Groups: 21
```

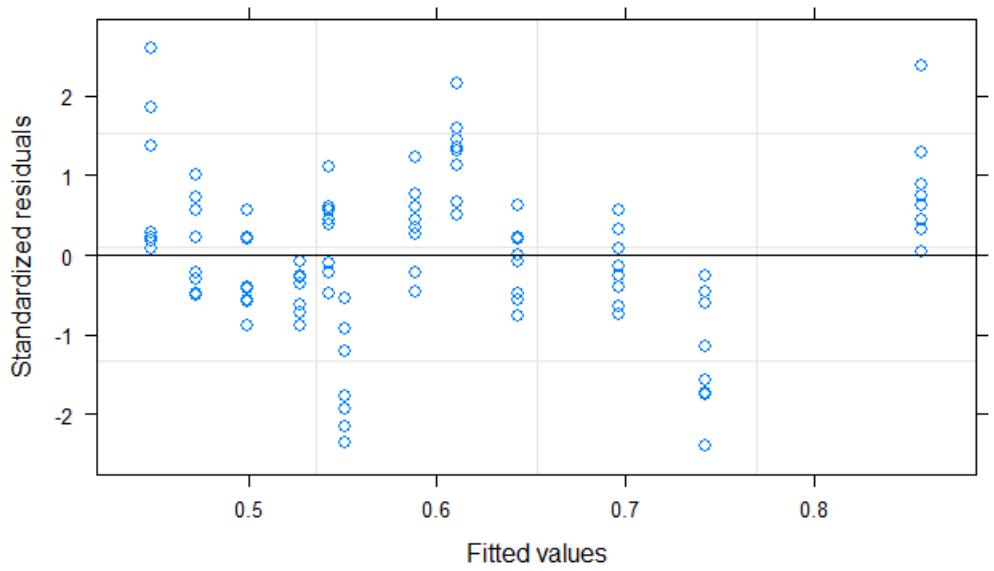


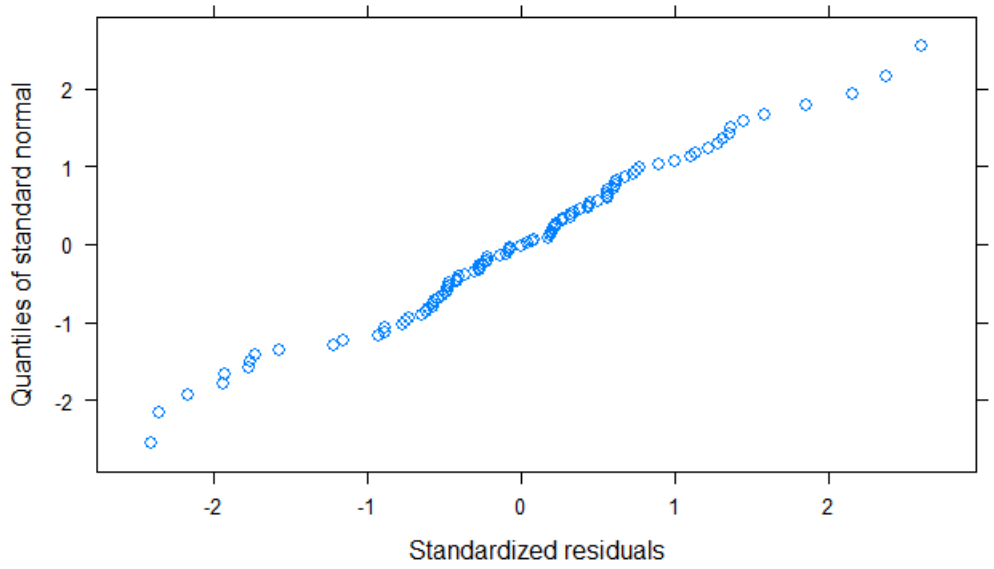


A2.6 LME model analysis comparing LDI data between male *ACE2*$^{-/y}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ groups

```
Linear mixed-effects model fit by REML
 Data: ACE2mal.LDI.data
        AIC       BIC   logLik
  -79.06461 -63.93388 45.53231
Random effects:
 Formula: ~1 | Mouse.ID
         (Intercept)  Residual
StdDev: 1.436911e-06 0.1281505

Fixed effects: Sqrt.LDI.ratio ~ Group * Timepoint
                     Value  Std.Error DF   t-value p-value
(Intercept)      0.9131619 0.03698819 78 24.687929  0.0000
Group           -0.1631579 0.05230920 14 -3.119105  0.0075
Timepoint       -0.0041629 0.00137276 78 -3.032475  0.0033
Group:Timepoint  0.0021892 0.00194138 78  1.127665  0.2629
 Correlation:
                (Intr) Group  Timpnt
Group           -0.707
Timepoint       -0.866  0.612
Group:Timepoint  0.612 -0.866 -0.707

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.03608617 -0.48888019  0.08230023  0.56239483  2.33320691

Number of Observations: 96
Number of Groups: 16
```

```
Fixed effects: LDI.ratio ~ Group * Timepoint
                    Value  Std.Error DF   t-value p-value
(Intercept)      0.8573152 0.05406692 78 15.856556  0.0000
Group           -0.2466602 0.07646218 14 -3.225911  0.0061
Timepoint       -0.0076644 0.00200661 78 -3.819593  0.0003
Group:Timepoint  0.0037065 0.00283778 78  1.306127  0.1953
 Correlation:
                (Intr) Group  Timpnt
Group           -0.707
Timepoint       -0.866  0.612
Group:Timepoint  0.612 -0.866 -0.707

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.40665763 -0.51661917  0.02387281  0.56663882  2.60947298

Number of Observations: 96
Number of Groups: 16
```




Quantiles of standard normal
2
1
0
-1
-2
-2
-1
0
1
2
Standardized residuals

A3. Supplementary Western blot information

Table A1. Table listing antibodies used for protein expression assays by Western blotting

Antibody		Species	Size of band detected (kDa)	Dilution in (TBS +0.05% tween)	Supplier and Catalogue #
Primary	GAPDH	Rabbit	37	1:10,000	Cell signalling Technology #5174
	TXNIP	Rabbit	60	1:1000	Cell signalling Technology #14715
	PGC1α	Rabbit	92	1:1000	Abcam #ab54481
	AMPKα	Rabbit	62	1:1000	Cell signalling Technology #5832
	Phospho-AMPKα (Thr 172)	Rabbit	62	1:1000	Cell signalling Technology #2535
	e-NOS	Rabbit	140	1:1000	Cell signalling Technology #9572
	Phospho-eNOS (Ser1177)	Rabbit	140	1:1000	Cell signalling Technology #9571
Secondary	Anti- rabbit IgG HRP conjugated	Goat		1:1000	abcam #ab6721
	Fluorophore conjugated anti-rabbit IgG (IRDye 800CW anti-rabbit IgG)	Donkey		1:15,000	Li-Cor #925-32213 (Millenium Sciences)

Table A2. Table listing primers used for mRNA expression assays by qRT-PCR

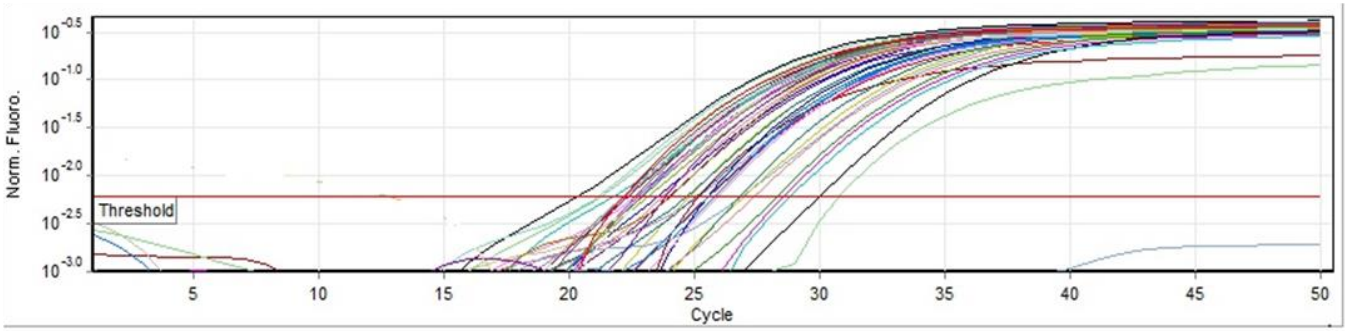
Gene	Species	Quantitect primer assay #	Supplier
AMPK	Mouse	QT00286923	Qiagen
TXNIP	Mouse	QT00296513	Qiagen
PGC1	Mouse	QT02524242	Qiagen
NOS3	Mouse	QT00152754	Qiagen
Gapdh	Mouse	QT01658692	Qiagen

A4. Repeatability results of SBP measurements

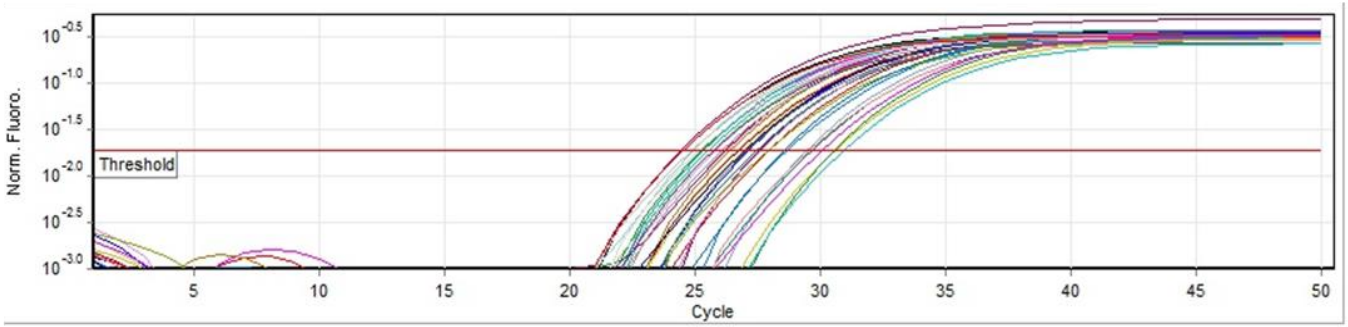
Table A3: Intra-observer repeatability results of SBP measurements

Mouse ID	Reading 1	Reading 2	Mean	Standard deviation
1	111.90	116.70	114.30	3.39
2	90.40	99.30	94.85	6.29
3	122.90	107.30	115.10	11.03
4	113.20	119.40	116.30	4.38
5	84.10	90.30	87.20	4.38
6	97.60	91.00	94.30	4.67
7	88.50	101.60	95.05	9.26
8	95.30	103.20	99.25	5.59
9	98.30	95.00	96.65	2.33
		Mean	**101.44**	5.70
		CoV %	5.62	

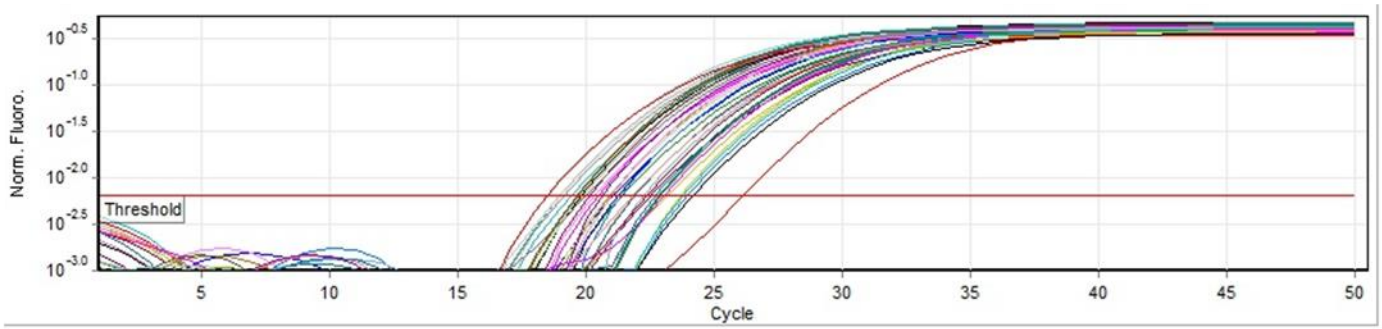
A



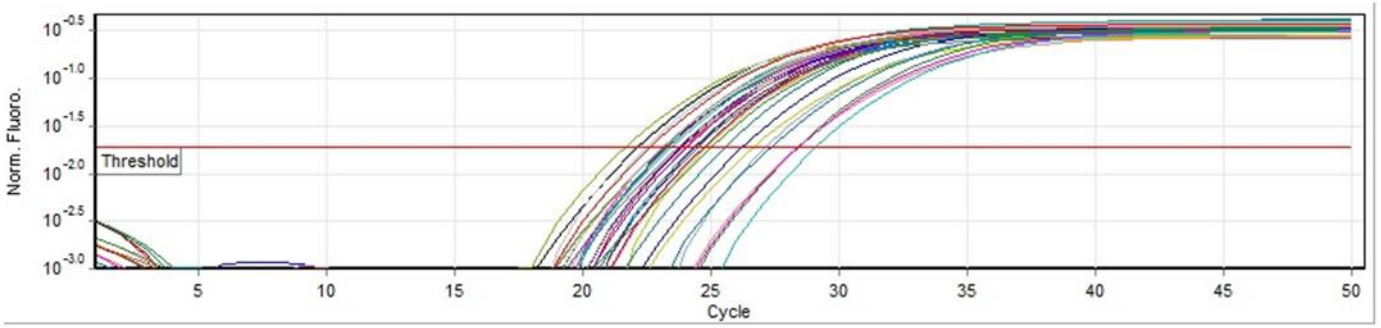
B



C


Norm. Fluoro.
Threshold
Cycle

D


Norm. Fluoro.
Threshold
Cycle

E

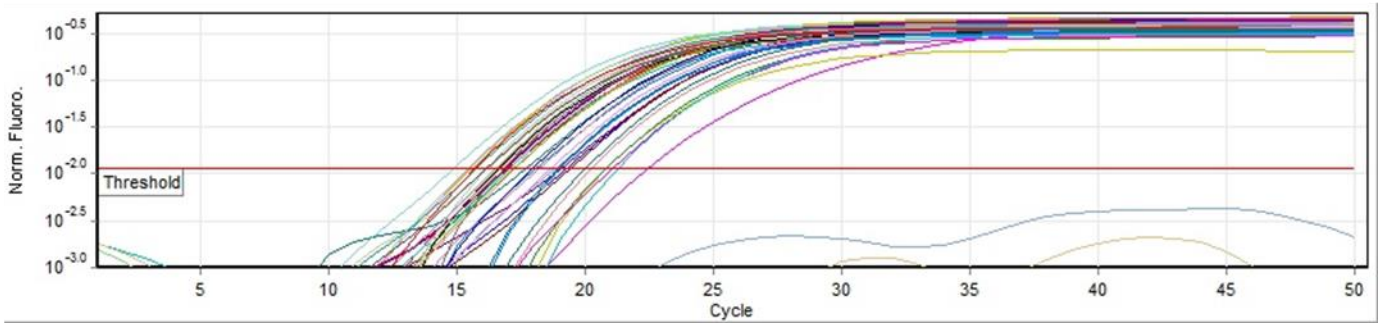

Norm. Fluoro.
Threshold
Cycle

Figure A1. Amplification plots of RNA from ischaemic gastrocnemius muscles of mice receiving metformin and vehicle control. A: Ampα1; B: Nos3; C: Txnip; D: Pgc1α; and E: Gapdh