THE EFFECT OF DIFFERENT FORMS OF EXERCISE ON THE CLINICAL, SYSTEMIC AND LOCAL BIOLOGICAL RESPONSES IN INTERMITTENT CLAUDICATION

Thesis submitted by

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SUMMARY

Consensus guidelines recommend that supervised exercise training (SET) should be made available as a treatment for adults with intermittent claudication (IC) on the basis of high quality evidence demonstrating improvement in walking performance following SET of sufficient intensity to induce claudication pain. Although treadmill-based SET is currently recommended, the optimal form of exercise is unknown and the impact of SET on the systemic and local biological responses of patients with IC has not previously been assessed.

The study presented in this thesis sought to determine, in adults with IC, whether 12 weeks of SET combining the additive effect of supervised interval based treadmill training with lower limb resistance exercises would facilitate a greater clinically meaningful response in the primary outcome of pain free walking distance (PFWD) compared with supervised treadmill based training alone. The study also assessed whether the potential of supervised interval based treadmill training with lower limb resistance exercise training to limit exposure to ischaemia reperfusion injury, would result in a more positive effect on secondary outcomes and markers of long term cardiovascular health compared with the recommended treadmill-based training regimen. These outcomes and markers included endothelial function, quality of life (QoL), body composition and systemic inflammatory burden.

The 6-minute walk test (6MWT) was used to assess PFWD. Markers of endothelial function were measured, including flow mediated dilatation, reactive-hyperemia peripheral arterial tonometry, serum nitric oxide (NO) and asymmetric dimethyl arginine (ADMA). Body composition was assessed using dual energy x-ray absorptiometry and proteomic analysis of the proteolytic calpain system was undertaken. Dietary intake and QoL were recorded using food frequency questionnaires and the Australasian Vascular QoL Index respectively and an array of inflammatory cytokines were assayed using enzyme-linked immunosorbent assay.

While neither exercise regimen was superior with respect to the primary outcome of PFWD, QoL improved in both groups. The treadmill-based SET resulted in a worsening of the biological marker of endothelial function (NO) and a relative loss of skeletal muscle mass which may be attributable to the increased level of activity of the proteolytic calpain enzyme. A combination based SET facilitated beneficial physiological effects including a gain in skeletal muscle mass, a relative reduction in calpain activity and a reduction in levels of the NO inhibitor ADMA.

The systemic inflammatory response to exercise in both groups was difficult to interpret, but raised the possibility that SET in patients with IC may induce a pro-inflammatory response, an adaptive anti-inflammatory response or an immunosuppressive response.

The study presented in this thesis demonstrates that treadmill-based exercise as a treatment for IC may be detrimental and challenge whether treadmill-based exercise training should remain the recommended treatment for patients with IC. The lack of significant improvement observed in walking performance means that the combination based SET cannot be recommended as a suitable alternative to a treadmill-based SET, however, with appropriate modifications, a combination based SET may have a role to play in the future. Further large-scale work is required to address the clinical need to look at better ways to design exercise programs for maximal benefit rather than harm. Consideration should also be given to long follow-up periods to facilitate the assessment of cardiovascular outcomes to ultimately determine whether or not the changes observed in the study presented in this thesis truly do manifest as detrimental to long-term health outcomes.

PUBLICATIONS/AWARDS ARISING FROM THIS RESEARCH

Refereed manuscripts

1. Delaney CL, Miller MD, Chataway T, Spark JI. A randomised controlled trial of supervised exercise regimens and their impact on walking performance, skeletal muscle mass and calpain activity in patients with intermittent claudication. *European Journal of Vascular and Endovascular Surgery* 2014;47(3):304-310.

2. Delaney CL, Miller M, Allan RB, Spark JI. The impact of different supervised exercise regimens on endothelial function in patients with intermittent claudication. *Vascular, 1708538114558329, first published on November 18, 2014*.

3. Delaney CL, Miller MD, Dickinson K, Spark JI. Change in dietary intake of adults with intermittent claudication undergoing a supervised exercise program and compared to matched controls. *Nutrition Journal* 2014, 13:100. http://www.nutritionj.com/content/13/1/100.

Conference abstracts

1. Delaney CL, Miller MD, Chataway T, Allan RB, Spark JI. Supervised exercise training for intermittent claudication: the clinical, systemic and local biological effect. 49th Surgical Research Society's (SRS) Annual Scientific Meeting. Adelaide South Australia November 9, 2012.
2. Delaney C, Miller M, Chataway T, Spark JI. Supervised exercise training and skeletal muscle mass in patients with intermittent claudication: a detrimental effect of the treadmill. 2013 Joint meeting of the Australian Vascular Biology Society and the Australia New Zealand Microcirculation Society. Barossa Valley September 5-8, 2013.

3. Delaney C, Miller M, Chataway T, Spark JI. Supervised exercise training and skeletal muscle mass in patients with intermittent claudication: a detrimental effect of the treadmill. Australia New Zealand Society of Vascular Surgery. Hobart – Tasmania Australia 12-15 October 2013.
4. Dickinson K, Delaney C, Spark JI, Miller M. Dietary intake of adults with intermittent claudication: A missed opportunity for delaying progression of peripheral artery disease? Dietitians Association of Australia National Conference, Brisbane, 15-17 May 2014.
5. Delaney C, Miller MD, Chataway T, Spark JI. A randomised controlled trial of supervised exercise regimens and their impact on walking performance, skeletal muscle mass and calpain activity in patients with intermittent claudication. European Society for Vascular Surgery Vascular Biology, Materials and Engineering Meeting, London UK, 16-17 May 2014.

Awards arising from research

1. Foundation for Surgery Australia and New Zealand Journal of Surgery Scholarship (2012).
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4. Foundation Daw Park Equipment Grant: The effect effect of different forms of exercise on the clinical, systemic and local biological responses in intermittent claudication. 2012. $10,000.
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DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Christopher L Delaney

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ABBREVIATIONS

6MWD: 6 minute walking distance

6MWT: 6 minute walking test

ABPI: Ankle Brachial Pressure Index

ACE: Angiotensin Converting Enzyme

ACR: Albumin Creatinine Ratio

ACSM: American College of Sports Medicine

ADL: Activity of Daily Living

ADMA: Asymmetric Dimethyl Arginine

AMDR: Acceptable Macronutrient Distribution Range

AUSVIQOL: Australasian Vascular Quality of Life Index

BODIPY-FL-casein: 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid labelled casein

CAD: Coronary Artery Disease

CAM: Cellular Adhesion Molecule

CCLI: Chronic Critical Limb Ischaemia

CLI: Critical Limb Ischaemia

CVD: Cardiovascular Disease

DDAH: Dimethylarginine Dimethylaminohydrolase

DEXA: Dual energy x-ray absorptiometry

DQES v2: Dietary Questionnaire for Epidemiology Studies version 2

DTT: dithiotreitol

ECG: Electrocardiogram

ED: Endothelial Dysfunction

EDTA: Ethylenediaminetetracetic acid

ELISA: enzyme-linked immunosorbent assays

F: Female

FFM: Fat free mass

FM: Fat mass

FMD: Flow Mediated Dilatation

GM-CSF: Granulocyte Macrophage Colony Stimulating Factor

GTN: Glyceryltrinitrate

HB: homogenisation buffer

HCl: hydrochloric acid

IC: Intermittent Claudication

ICAM: Intra-Cellular Adhesion Molecule

IFN: Interferon

IL: Interleukin

IRI: Ischaemia Reperfusion Injury

ITT: intention to treat

Kcal: kilocalorie

KCl: potassium chloride

LDL: Low Density Lipoprotein

M: Male

MI: Myocardial Infarction

MIMIC: Mild to Moderate Intermittent Claudication

MOS-SF-36: Medical Outcomes Study Short Form 36

MWD: Maximum Walking Distance

MWT: Maximum Walking Time

NE: Neutrophil Elastase

NICE: National Institute for Health and Clinical Excellence

NLR: Neutrophil Lymphocyte Ratio

NO: Nitric Oxide

NOS: Nitric Oxide Synthetase

PAD: Peripheral Arterial Disease

PAT: Peripheral Arterial Tonometry

PECAM: Platelet Endothelial Cellular Adhesion Molecule

PFWD: Pain Free Walking Distance

PFWT: Pain Free Walking Time

PMSF: phenylmethylsulfonyl fluoride

QALY: Quality Adjusted Life Year

QOL: Quality of life

RCT: Randomized Controlled Trial

REACH: Reduction of Atherothrombosis for Continued Health

REE: Resting Energy Expenditure

RHI: Reactive Hyperaemia Index

RH-PAT: Reactive Hyperemia Peripheral Arterial Tonometry

ROS: Reactive Oxygen Species

RQ: Respiratory Quotient

SAA: Serum Amyloid A

SACREC: Southern Adelaide Clinical Research and Ethics Committee

SD: Standard Deviation

SDT: Suggested Dietary Target

SET: Supervised Exercise Training

SMC: Smooth Muscle Cell

SMM: Skeletal Muscle Mass

TASC: Trans-Atlantic Inter-Society Consensus

TF: Tissue Factor

TGF: Transforming Growth Factor

TNF: Tumour Necrosis Factor

VCAM: Vascular Cellular Adhesion Molecule

VEGF: Vascular Endothelial Growth Factor

VSMC: Vascular Smooth Muscle Cell

CHAPTER 1: INTRODUCTION

1.1 Overview of Peripheral Arterial Disease

1.1.1 Definition of Peripheral Arterial Disease

Peripheral arterial disease (PAD) is an occlusion or stenosis of an artery, usually one belonging to the leg or arm. It is typically thought of as a local manifestation of the diverse pathophysiological processes associated with the systemic disease state atherosclerosis, however auto-immune disorders, aneurysmal disease and thromboembolism may result in occlusion, thrombosis or stenosis of an artery and can be broadly defined as PAD (1). In addition, anatomical factors may contribute to cases of PAD specific to the arteries in the arm (e.g. thoracic outlet syndrome) or the leg (eg popliteal entrapment syndrome) (2).

1.1.2 History of Peripheral Arterial Disease

The nature of the circulatory system was first described by Harvey in the early seventeenth century and gangrene was first recognised as a manifestation of arterial disease by Quesnay in 1771 (3). Subsequently, Sir Benjamin Brodie first described the syndrome of intermittent claudication in man in 1846, while Sir Thomas Lewis demonstrated that claudication pain was due to a build-up of pain provoking metabolites rather than “arterial spasm” as had previously been speculated (3). Following this, visualisation and haemodynamic assessment of arterial stenosis evolved from the primitive equipment which facilitated tonoscillography (measurement of arterial pressure wave amplitude via transmission to a piezo-electric manometer and amplification to a pen-recorder) and translumbar aortography, to the modern technology utilised for digital subtraction angiography. From a therapeutic perspective, Hippocrates recommended amputation for the treatment of gangrene in the fifth century Before Christ. The use of autologous vein to replace or bypass diseased arteries was reported

by Alexis Carrel in the early 1900's (4), while the first percutaneous transluminal angioplasty for the treatment of PAD was performed by Charles Dotter in 1964 (5).

1.1.3 Diagnosis of Peripheral Arterial Disease

A concise clinical history and examination focusing on risk factors, walking impairment, symptoms of ischaemic rest pain and inspection of peripheries for non-healing wounds, pulses and other trophic changes is critical for a diagnosis of PAD. Given the variable presentation of the disease, it has recently been shown that clinicians who utilise a classic history of walking impairment alone to diagnose PAD are likely to miss 85-90% of cases (6). Once history and examination have both been used to screen patients likely to be at risk of PAD, measurement of ankle brachial pressure index (ABPI) can be undertaken to support the diagnosis. This index is a simple, non-invasive, bedside test to measure the systolic blood pressure (mmHg) in both brachial arteries and in the posterior tibial and dorsalis pedis arteries in both ankles. The ABPI for each ankle artery will be the systolic blood pressure of that artery divided by the higher of the two brachial artery pressures as illustrated in *Figure 1*. An ABPI of <0.9 has a sensitivity of 90% and specificity of 98% for a stenosis of 50% or more in a major leg artery defined by angiography (6). The ABPI provides information about severity of disease and can also provide important information regarding the extent of systemic atherosclerotic burden and subsequent overall cardiovascular risk (1). An ABPI of <0.9 carries a 3-6 fold increased risk of cardiovascular mortality (7) and is an independent risk factor for cardiovascular events and all cause mortality (8). Statistically lower survival rates are associated with progressively lower ABPI's (8, 9). An index of >1.3 is deemed abnormally high and is reflective of calcification within a vessel wall preventing adequate compression and pressure measurement (10). Such a level has also been demonstrated to be associated with higher rates of mortality (9). Additional non-invasive diagnostic testing can be performed in these patients to confirm a diagnosis of PAD. These include toe photo-

plethysmography, trans-cutaneous oxygen measurements, and duplex ultrasound imaging. Ultrasound is one of the most common non-invasive techniques used to define anatomy, haemodynamics and lesion morphology.

Once a diagnosis of PAD has been established, more invasive imaging (either catheter directed digital subtraction angiography, magnetic resonance angiography or computed tomography angiography) can be undertaken to provide the treating clinician with information on the anatomical location and extent of disease, thus allowing appropriate treatment strategies to be formulated.

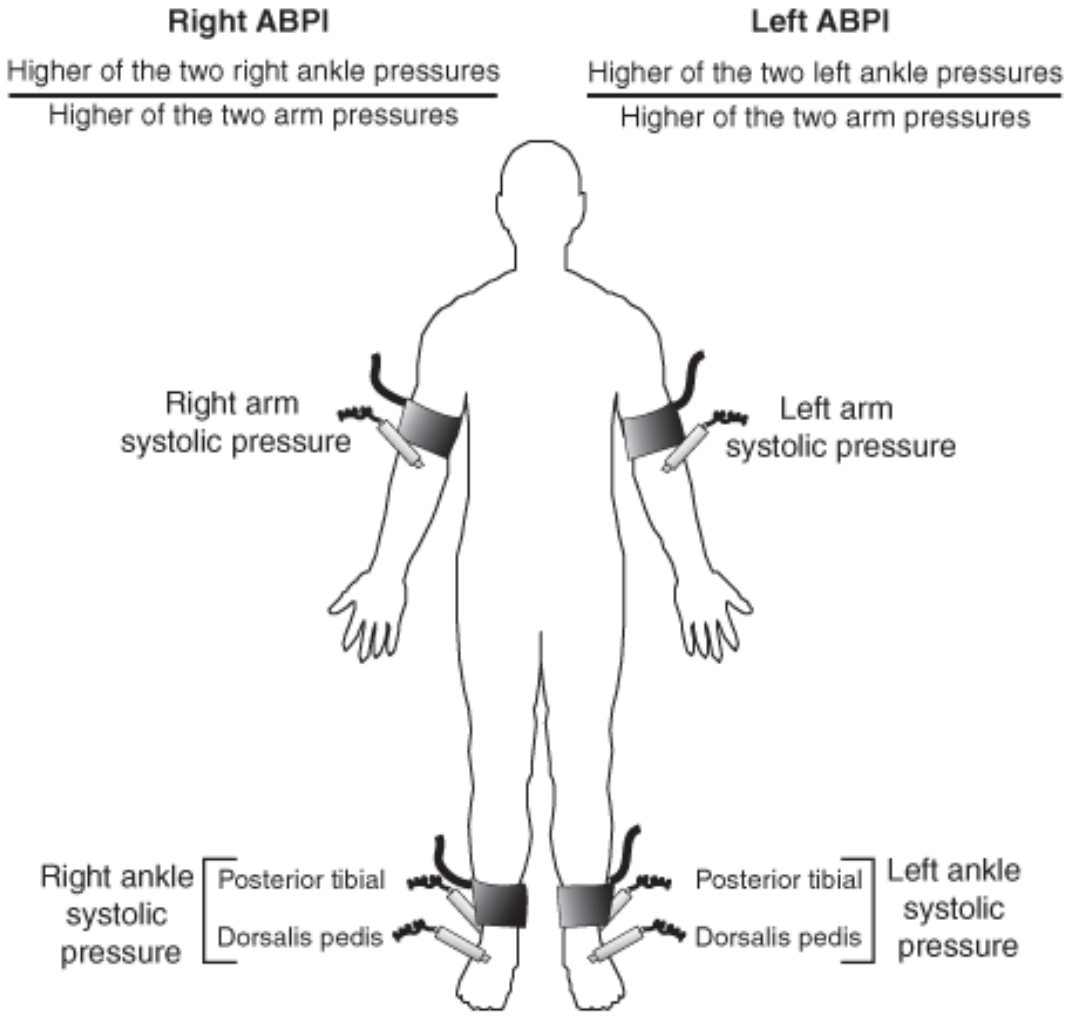

Figure 1: Measurement of the ankle brachial pressure index (1)

1.1.4 Epidemiology of Peripheral Arterial Disease

Peripheral arterial disease is a major public health problem worldwide. While it is widely accepted that the incidence of PAD increases with age, the heterogeneous nature of the population based studies designed to assess the epidemiology of PAD makes accurate estimations of incidence and prevalence challenging. Highlighting this, is the fact that estimates of PAD prevalence in the United States have ranged from 3%-30% (11).

The greatest variation was noted in the diagnostic criteria of PAD, with the cut-off value for abnormal ABPI's ranging from <0.8-<0.95 (12, 13). Furthermore, such diagnostic values do not allow for the potential falsely elevated ABPI readings that can be seen in diabetic patients, due to the heavy vascular calcification associated with the disease process. These patients are often subsequently found to have quite severe PAD. Several studies, notably the widely cited PARTNERS study (6) actively recruited diabetic patients to their cohort, potentially resulting in an under-estimation of PAD prevalence. Other studies included patients for screening only if they were deemed to have one or more risk factors for vascular disease (as determined by questionnaires or medical records) (6, 12, 13). This should not be considered as optimal population based sampling and the bias introduced as a result of this may have led to a significant over-estimation of the prevalence of PAD cited in these papers. Perhaps the most reliable, large scale population based study to date is from Selvin et al (14), who assessed 2174 individuals >40 years of age from the general population of the United States, with or without risk factors for vascular disease. PAD was defined as an ABPI <0.9 in either leg. Within the study population, overall prevalence of PAD was 4.3%, increasing to 14.5% in individuals aged >70 years. Interestingly, >95% of individuals diagnosed to have PAD had one or more vascular risk factors, while the prevalence of PAD was significantly higher in those with black ethnicity (7.8%) compared with Caucasians (4.4%). This finding is supported by Kullo et al's (15) study on ethnic differences in PAD who found in 453 African-

Americans and 478 Caucasian Americans that African-American ethnicity was associated with lower ABPI's and an increased prevalence of PAD.

The results from Selvin et al (14) are comparable to a similar large-scale (n=4470) population based Australian study from Fowler et al in 2002 (16). Including only men aged between 65-83 years, Fowler et al reported a prevalence of PAD of 16.6% (16). The absence of women from this study is unlikely to affect the reported prevalence, as the majority of the large scale population based studies referred to above would lead us to believe that gender does not confer a significant difference in the prevalence of PAD (6, 14).

In summary, using an ABPI of <0.9 as diagnostic of PAD, it seems that total disease prevalence is in the range of 3-10%, increasing to 15-20% in those aged >70 years (7). Significantly, despite the heterogeneity of the literature, it seems clear that only about 20% of patients with PAD are symptomatic. The need to identify the 80% of patients with asymptomatic disease becomes relevant when one considers the natural history of PAD (*See section 1.1.8* Natural history and prognosis of Peripheral Arterial Disease*).*

1.1.5 Economic Burden of Peripheral Arterial Disease

Despite the statistics demonstrating the magnitude of the public health problem created by PAD, there remains limited knowledge in Australia regarding the economic burden of the disease. The most recent and seemingly the only population based data available are from 1994, when the direct health care cost of PAD in Australia was $180 million, of which 78% was associated with hospitalisations (17). While this represented <1% of the total CVD health expenditure at the time (≈ $5billion), this figure only corresponded to limb morbidity associated with PAD and failed to capture the vast expenditure related to cardiovascular events indirectly associated with PAD and the systemic atherosclerotic disease state (17). Perhaps the economic implications of PAD are more accurately identified by Ademi et al

(18), who extracted one-year follow-up data from Australian participants enrolled in the REACH (Reduction of Atherothrombosis for Continued Health) Registry and assigned costs to each health care item (pharmacotherpaies, hospitalisations, outpatient appointments, imaging studies) based on Australian Government reimbursement data. Of the 2873 participants, 69 were identified as having PAD without clinical evidence of vascular disease in other territories. The estimated annual health care cost associated with treatment of these patients was AU$16,602 per person with disease (18). If this figure is extrapolated to reflect the current Australian population of approximately 24 million (19), with a conservative estimate of 5% of people affected by PAD (based on epidemiological data above), then greater than AU$11 billion is spent annually on PAD. A similar calculation was performed by a research and consulting company in the United States (US) using US REACH data. They concluded that in 2010, PAD cost the US between US$164 and 290 billion (20). Given that the population of the US is almost 15 times that of Australia, these financial figures are almost equivalent on a cost per person basis.

1.1.6 Classification of Peripheral Arterial Disease

Lower extremity PAD is the focus of this thesis, the manifestations of which are due to stenoses or occlusions within the arterial tree of the leg, causing a reduction in blood flow to the leg, leading to varying degrees of tissue ischaemia. This can occur acutely (ie onset of symptoms is <2 weeks) resulting in a constellation of signs and symptoms commonly referred to as “the 6 P’s” (pain, pallor, poikolothermia, pulselessness, parasthesia and paralysis) and may be classified by Rutherford’s classification of acute limb ischaemia into viable limb ischaemia, marginally threatened limb ischaemia, immediately limb threatening ischaemia or irreversible limb ischaemia (7). More commonly, PAD is chronic in nature (symptoms present for >2 weeks). The most frequent symptom of chronic PAD is intermittent claudication (IC) (1, 7). Derived from the latin word *claudicare – to limp*, IC is

defined as walking induced pain and cramping in one or both legs that is relieved by rest. The level of pain associated with IC can be mild to extremely severe. Intermittent claudication is most common in calf muscles but can affect the feet, thighs, hips or buttocks.

At the severe end of the spectrum, patients with PAD present with ischaemic rest pain and may develop gangrene or ulceration (critical limb ischaemia). Significantly, epidemiological studies suggest that for every one patient with symptomatic PAD there are 3-4 patients with asymptomatic disease (7), of whom approximately 1/3 have a complete occlusion of a major lower limb artery (21).

The spectrum of disease severity that represents chronic PAD has historically been described by Rene Fontaine back in 1934 and more recently by Rutherford. These criteria separate PAD into objective categories on the basis of the severity of signs and symptoms such as IC, rest pain or the presence of arterial ulceration/gangrene. Rutherfords system of classification is referred to in this thesis and can be seen in *Table 1*.

Classification of PAD may also be based on anatomical distribution of disease. Broad anatomical definitions of PAD include “aorto-iliac disease” and “infra-inguinal disease”. The latter could be further divided into “femoro-popliteal” and “infra-geniculate” (below knee) disease. More complex classifications also exist. The Trans-Atlantic Society Consensus (TASC) classification (*see Figure 2*) was designed to provide some indication of the site, extent and distribution of disease as well as to guide choice of treatment (7, 22). Specific to femoro-popliteal disease, the TASC classification shown in *Figure 2* ranges from Type A lesions, defined as a “single stenosis <10cm in length or a single occlusion <5cm in length” to Type D lesions defined as “chronic total occlusions of the common or superficial femoral artery (>20cm, involving the popliteal artery) or chronic total occlusion of popliteal artery

and proximal trifurcation vessels". A similar classification system has been designed by TASC relevant to aorto-iliac disease.

Table 1: Rutherford's classification of chronic Peripheral Arterial Disease

Category	Clinical
0	Asymptomatic disease
1	Mild claudication
2	Moderate claudication
3	Severe claudication
4	Ischaemic rest pain
5	Minor tissue loss (ischaemic ulceration not exceeding ulcer of digits of foot)
6	Major tissue loss (extending proximal to transmetatarsal level)

1.1.7 Risk factors for Peripheral Arterial Disease

The complex nature of atherosclerosis and its manifestation as PAD means that there are a number of associated risk factors. Without doubt the most significant risk factors predisposing to the onset and progression of PAD are smoking and diabetes, with evidence suggesting a 3-4 fold increase in risk of PAD associated with each of these factors independently, when compared to non-smokers and non-diabetics (7). Furthermore, in diabetics, every 1% increase in haemoglobin A1c has been found to correspond with a 26% increase in risk of PAD (23).

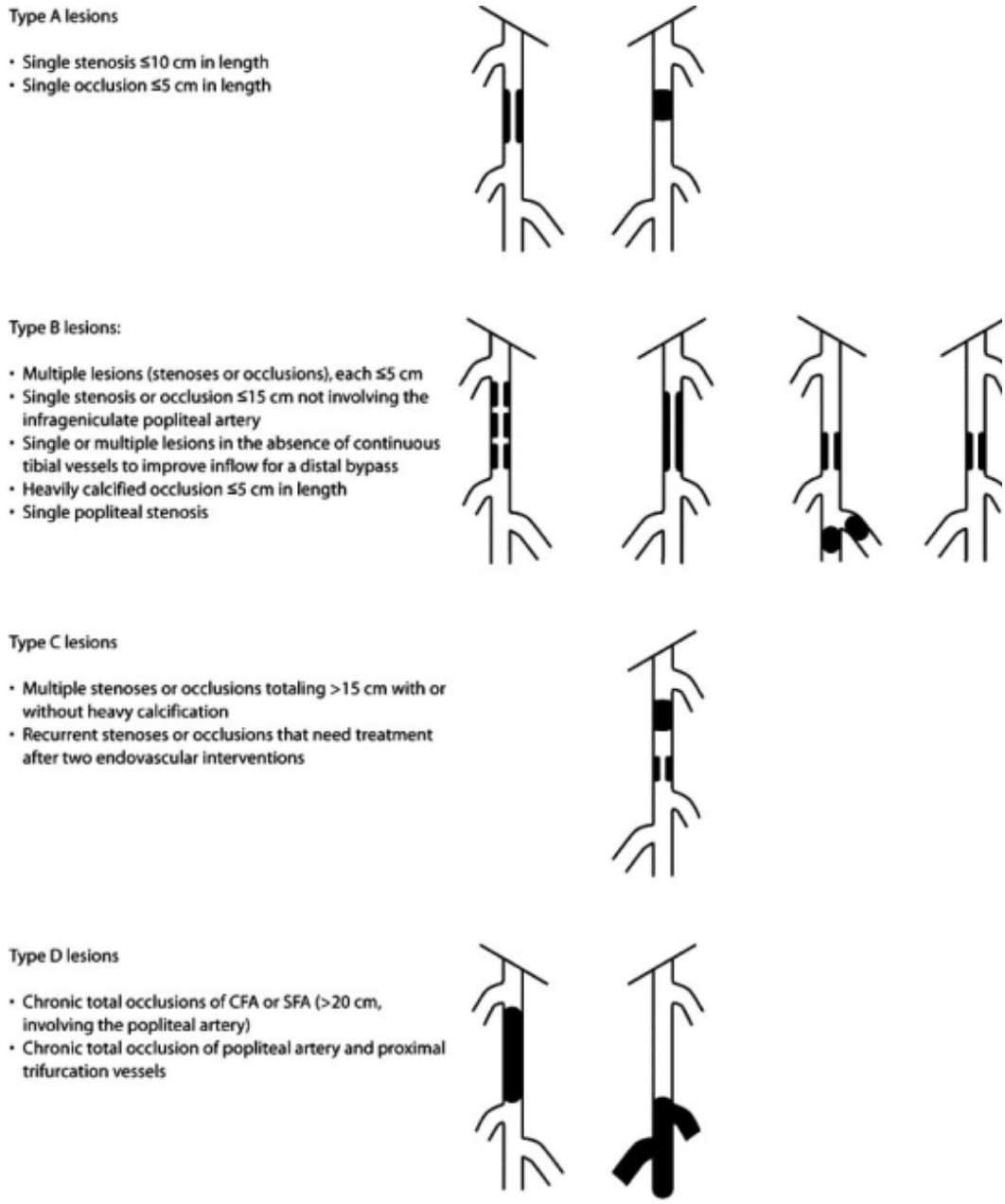

Figure 2: TASC classification of femoro-popliteal lesions (22)

Similar to other manifestations of atherosclerosis, hypertension and dyslipidemia and raised levels of the relatively poorly understood lipoprotein-a, are also known to be associated with PAD, as is increasing age (1, 7). Most recently, evidence supporting a genetic link and heritable basis of PAD has been provided by data from numerous genetic association studies

(24), although no single gene has been identified and it seems likely that PAD results from hundreds of genes interacting with themselves and the environment to cause disease.

Other factors associated with PAD include the presence of obesity (defined as body mass index >30kg/m^2), as well as hyperviscosity and hypercoagulability, both of which are known to be markers of poor prognosis in PAD (7). Associations have also been made with chronic renal insufficiency (25) and hyperhomocysteinemia due to the tendency of homocysteine to promote oxidant stress-induced cellular toxicity (7).

1.1.8 Natural history and prognosis of Peripheral Arterial Disease

Peripheral Arterial Disease is thought of as a local manifestation of the systemic disease process atherosclerosis and PAD is therefore a marker for advanced cardiovascular disease (CVD) involving coronary, cerebral, renal and aortic vessels. In fact, it has been shown that regardless of symptomatology up to 60% of patients with PAD have concurrent coronary artery disease (CAD) and/or cerebrovascular disease (10), while up to 40% of patients have haemodynamically significant (>50% stenosis) renovascular disease (7). The natural history of PAD is therefore characterised by an increased risk of CVD events, occurring at a much higher frequency than complications directly arising from PAD. Such an increased risk is directly related to the severity of PAD as measured by ABPI's and symptomatology and it is therefore important to consider patients with critical limb ischaemia (CLI) as distinct from those with asymptomatic disease or IC.

Excluding those with CLI, PAD patients have a 2-3% annual incidence of non-fatal myocardial infarction (MI) with a 2-3 fold increased risk of ischaemic heart disease as compared to an age-matched population (7). At 5 years after initial presentation, 20% of these patients will have had a non-fatal cardiovascular event (MI or stroke), while 10-15% will be deceased, 75% of these from cardiovascular causes (7). With respect to lower-limb

complications, about 75% of patients will remain stable requiring only lifestyle modification and medical management of risk factors, 20% will develop lifestyle limiting symptoms requiring revascularisation, while 5% will progress to limb threatening CLI (7). Diabetes and smoking are the risk factors most likely to predict this disease progression (7) and for those with CLI, 25% will be dead and 30% will require amputation within 12 months of diagnosis (7).

1.1.9 Treatment of Peripheral Arterial Disease

Treatment strategies for patients with PAD are twofold: (1) Modification of risk profile aimed at preventing disease progression and minimising risk of future CVD events; (2) Improving symptomatology and functional status.

1.1.9.1 Strategies for risk factor modification

Over the last 10-20 years, the concept of "Best Medical Therapy" has evolved for the treatment of PAD. This comprises smoking cessation, improved glycaemic control and the use of pharmacotherapies including antiplatelet agents, anti-hypertensive medications and statins. Such a concept is evidence based and has been found to significantly reduce risk of future cardiovascular events as well as delay PAD progression and contribute to improvements in symptomatology. Despite this and the fact that PAD is considered an equivalent to coronary artery disease, PAD as a disease process is not well recognised by primary care physicians and patients with PAD are currently undertreated with respect to atherosclerotic risk factor control compared to those with coronary artery disease.

Given the importance of smoking as a risk factor for PAD, strong and repeated smoking cessation advice should be provided to patients. Evidence suggests that greater compliance with such advice can be achieved through the concurrent use of nicotine replacement therapy, cognitive behavioural therapy and anti-depressant therapy (7). Recent guidelines published

by the United Kingdom National Institute for Health and Clinical Excellence (NICE) report a halving in excess cardiovascular risk after one year of smoking cessation, with an equivalent risk as non-smokers five years after successful cessation (26). Furthermore, smoking is associated with a 3-fold increase in failure of lower extremity bypass grafts, while smoking cessation restores patency towards that of patients who have never smoked (27). Despite this, in the PARTNERS study (n=6979), designed to assess the intensity of risk factor modification in the primary care setting, smoking cessation therapies were only prescribed to approximately 50% of patients with diagnosed PAD (6).

Like smoking, the presence of diabetes is also associated with a significant risk of PAD (25). It is well established that the incidence of major limb amputation is greater in diabetics due to the associated presence of peripheral neuropathy and decreased resistance to infection (28), however, the role of improved glycaemic control relative to PAD and CVD is unclear. Despite this, TASC II guidelines recommend aggressive control of blood glucose levels with a target Haemoglobin A1c of <7% (7). This is based on evidence arising from the UK Prospective Diabetes Study, which included 5102 patients with newly diagnosed type II diabetes. Aggressive blood glucose control (Haemoglobin A1c <7%) in these patients did not result in improvement in symptoms of PAD but a reduction in the incidence of diabetic related retinopathy and peripheral neuropathy (11).

Antiplatelet agents inhibit platelet aggregation and activity at sites of vascular damage such as atherosclerotic plaque rupture and are therefore critical in the primary and secondary prevention of thrombotic cardiovascular events (29). A recent large meta-analysis reported a near 25% reduction in CVD events through the use of anti-platelet agents (predominately aspirin) in patients with PAD (29). Additional beneficial pleiotropic effects of aspirin have also been proposed including an anti-inflammatory effect and ability to inhibit plaque growth and vascular smooth muscle cell proliferation (30). The relatively low cost of aspirin means

that it is currently the anti-platelet agent of choice, however, the emerging concept of aspirin resistance and inability of some patients to tolerate its associated gastro-intestinal side-effects means that it is not effective in 20-40% of patients (26). In these patients, a newer antiplatelet agent, clopidogrel, is recommended. Although lacking the pleiotropic effects of aspirin, clopidogrel has an equivalent safety profile to aspirin and when compared to aspirin, has been shown to further reduce the risk of fatal or non-fatal myocardial infarction or stroke by 8.7% in all patients and by 23.8% in patients with PAD (31). Unfortunately, the relative cost-benefit analysis of clopidogrel is somewhat prohibitive for its use as a first line agent.

Management of lipid levels in patients with PAD is also important. The most common lipid derangements in patients with PAD are increased levels of triglycerides and low levels of high density lipoproteins (the so called "good cholesterol" due to its antioxidant/anti-inflammatory effects and ability to efflux cholesterol). The first large-scale randomised controlled trial to assess drug therapy for hyperlipidemia (32) demonstrated that in 4444 patients with symptomatic CAD, treatment with statins was safe and improved survival. Subsequently, in a large-scale randomised controlled trial, the use of statin was observed to reduce CVD events by almost 20% over 5 years, compared with placebo in PAD patients (33). Significantly, the pleiotropic effect of statins means that the observed reduction in CVD event rate was achieved irrespective of whether the baseline lipid status was normal or impaired. Such pleiotropic effects include improving endothelial function, enhancing the stability of atherosclerotic plaques and possibly causing plaque regression if used in high dose, decreasing oxidative stress and inflammation and inhibiting the thrombogenic response (34). All patients with a diagnosis of PAD should therefore be prescribed statin therapy, with the rare exception of those who develop liver failure or statin-induced myopathy.

Anti-hypertensive medications are the third class of drug that should routinely be prescribed for those with PAD. Regardless of choice of anti-hypertensive, adequate treatment of

hypertension has been found to significantly reduce the risk of future CVD event with a benefit independent of ABPI (7). The American Heart Association recommend a blood pressure of <140/90mmHg or <130/80mmHg for those with diabetes of renal insufficiency (35). The latter recommendation is supported by findings from the Appropriate Blood Pressure Control in Diabetes (ABCD) trial that demonstrated intensive blood pressure control (mean=133/78mmHg) versus moderate blood pressure control (mean=139/86mmHg) resulted in a reduction in diabetic complications, CVD events and all-cause mortality at 5 years (36). Also in the ABCD trial, those patients treated with enalapril [an angiotensin converting enzyme inhibitor, (ACE-inhibitor)] compared with nisoldipine (calcium channel blocker) were found to have significantly fewer myocardial infarctions. Furthermore, the ACE-inhibitor was associated with preservation of renal function in both intensive and moderate blood pressure control groups. Similar findings were reported from the Heart Outcomes Protection (HOPE) study, who compared the effects of the ACE-inhibitor ramipril to placebo in 3577 patients with diabetes and at least one other cardiovascular risk factor. Beneficial effects of ramipril on CVD events and all-cause mortality were identified which persisted independent of blood pressure control (37).

Significantly, like the pleiotropic effects of aspirin and statins, ACE-inhibitors have been demonstrated to have benefit in PAD patients independent of blood pressure control. Evidence suggests anti-proliferative and direct anti-atherogenic effects of ACE-inhibitors, as well as a beneficial anti-oxidant effect and stimulation of increased nitric oxide production with subsequent improvement in endothelial function (38). These additional cardio-protective benefits mean that, in the absence of contraindications, ACE-inhibitors should be considered as first line treatment for PAD patients. Most recently the role of the ACE-inhibitor ramipril has also been linked with symptomatic improvements in patients with IC. Specifically, in a

trial of 33 participants, 24 weeks of treatment with ramipril resulted in significant improvement in walking performance compared with placebo (39).

The implementation of "best medical therapy" is an essential part of the management strategy for any patient with PAD and is particularly focused upon modification of risk profile to prevent or reduce the incidence of future CVD events and delay progression of the disease process. While some components of "best medical therapy" have been linked to improvement in symptoms of PAD patients, there are other treatment strategies with a specific purpose of providing symptomatic improvement.

1.1.9.2 Pharmacotherapy to provide symptomatic improvement in PAD and IC

Several drugs have been proposed to provide symptom relief to PAD patients, particularly those with IC, however, there are very few drugs with direct evidence of clinical utility. Cilostazol is a phosphodiesterase III inhibitor which exerts anti-platelet and vasodilatory effects (40). Meta-analyses have confirmed a beneficial effect on walking performance in patients with IC, however, this effect is only modest (50-70m) and it is often poorly tolerated due to associated side-effects such as headaches (30%), diarrhoea (15%) and palpitations (9%) (40). Cilostazol has a level IA recommendation from the American Heart Association for the treatment of IC (41).

Administration of the naturally occurring quaternary ammonium compound carnitine has also been shown by some authors to provide improvement in walking performance in patients with IC (42, 43). Carnitine plays a key role in the transfer of the energy source acyl-coenzyme A, an intermediate metabolic product of fatty acids and carbohydrates, across the mitochondrial membrane where it is oxidised in the Krebs cycle to produce energy (44, 45). In patients with IC, transient ischaemia induced by exercise may result in a state of metabolic stress, leading to an alteration in carnitine metabolism (46). As a result, carnitine levels and

skeletal muscle energy supplies are depleted, potentially limiting exercise capacity. It has been proposed that restoration of muscle carnitine stores enables the correction of carnitine metabolism and is the mechanism by which carnitine supplementation improves walking performance in patients with IC (47). A recent systematic review suggests that the benefit of carnitine supplementation on walking performance of those with IC may be superior or approaching equivalence to current therapies (48).

Other drugs have been trialled, including pentoxifylline which lowers blood viscosity and has a grade IIb recommendation from the American Heart Association for the treatment of IC. L-arginine has the ability to enhance endothelial function through up-regulation of NO expression, however, there is insufficient evidence of clinical benefit to recommend its use in the treatment of IC (7, 26).

Given the, at best, modest improvement in symptoms (ie walking performance) and potential side-effect profile associated with the use of drugs such as cilostazol, they are rarely prescribed as a first line treatment by clinicians in Australia. Instead, in order to improve the symptoms of PAD some patients are offered an intervention in the form of an open surgical or endovascular revascularisation procedure.

1.1.9.3 Interventions to provide symptomatic control in PAD and IC

Endovascular therapy is a form of minimally invasive surgery which involves the percutaneous introduction of a catheter into a blood vessel, typically the femoral artery, using seldinger technique. This facilitates the treatment of occlusive atherosclerotic lesions using methods such as balloon angioplasty or stenting, subsequently improving blood flow to the muscles and other tissues in the leg and foot and in doing so, improving symptoms of PAD, functional capacity and quality of life.

Open surgery for the treatment of PAD typically involves arterial bypass and/or endarterectomy (removal of atherosclerotic plaque from inside the artery). When compared with endovascular techniques, it has the disadvantage of requiring general or regional anaesthesia rather than local anaesthesia, longer recovery times and increased short-term morbidity and mortality. Open surgery does however, offer greater durability compared with endovascular procedures, which often require multiple procedures for repeat revascularisation.

Typically, the indications for intervention include acute limb ischaemia, chronic critical limb ischaemia (CCLI) with rest pain or tissue loss and lifestyle or economically limiting IC in patients who have failed to respond to conservative management strategies employing "best medical therapy". The decision on choice of revascularisation technique (open or endovascular) is complex. Consideration must be given to factors which increase the risk of limb loss, including comorbid factors such as smoking and diabetes mellitus, as well as poor cardiac output and the presence of ulceration or skin breakdown. Patient and physician preferences are also important, as is the concept of risk associated with any invasive procedure. Blood loss, infection, wound and cardio-respiratory complications can be associated with any surgical procedure. Specific to revascularisation techniques is the risk that graft or stent thrombosis may occur either acutely or after several months to years which can result in acute on chronic ischaemia and immediate limb compromise. The same may be the case if thrombo-embolic trashing was to occur in association with an endovascular procedure.

In patients with CCLI, such a risk is not so relevant given that the severity of their condition generally mandates major amputation unless revascularisation is undertaken. In those with IC however, the likelihood of progression to limb threatening ischaemia is low so the risk of limb loss in association with a therapeutic procedure takes on far more significance.

The anatomical location and burden of atherosclerotic disease means that in some cases endovascular therapies are not suitable and open surgery is the only option. The classification system described by TASC *(see Figure 2 and section 1.1.6* Classification of Peripheral Arterial Disease*)* can be used to guide such decision making. On the basis of such classification, the TASC II consensus recommends endovascular revascularisation for Type A lesions and surgery for Type D lesions. Endovascular treatment is suggested to be preferable for Type B lesions and surgery for "good-risk" patients with Type C lesions (7).

Results of a recent study from Murphy et al (49) demonstrate why many clinicians continue to take a conservative approach in the management of IC. This multi-centre trial randomised participants with haemodynamically significant aorto-iliac disease and associated moderate to severe claudication to either "best medical therapy" alone or in combination with either endovascular stenting or supervised exercise training (SET). After six months, it was found that SET offered greater improvement in walking performance than stent revascularisation, without the risks associated with an invasive procedure.

1.1.9.4 The role of supervised exercise training in IC

A very important concept in the treatment of IC is SET. This is a non-invasive treatment strategy that consensus guidelines recommend should be made available as the first line of treatment for all adults with PAD and IC (7, 26). The consensus guidelines were established on the basis of extensive high quality evidence demonstrating improvement in both pain free and maximum walking performance following SET of sufficient intensity to induce claudication pain. The effect of different forms of supervised exercise training on the clinical, systemic and local biological responses in patients with IC is the focus of this thesis.

1.2 Role of the Vascular Endothelium – Cellular Mechanisms

The vascular endothelium is a monolayer of cells acting as a barrier between blood and the pro-thrombogenic vessel wall (50). Once thought to be inert, the vascular endothelium is in fact a dynamic organ that plays a key role in vascular homeostasis and systemic well-being through a complex interplay of interactions between endogenous biochemical and biomechanical signals and the secretory potential of the endothelial cells themselves (50). The selective permeability of the endothelium allows for the movement of nutrients, macromolecules and selected cells between the lumen and surrounding tissues (51), while it provides intrinsic regulation of inflammation, coagulation, vasomotor tone, angiogenesis and smooth muscle proliferation (51, 52). Endothelial dysfunction (ED) is a state of impairment of these regulatory functions, characterised by an impaired endothelium dependent vasodilatory response to changes in flow or stimuli and a systemic pro-inflammatory and pro-thrombotic state (53, 54). It has been proposed as the mechanism linking cardiovascular risk factors to established disease and its onset has been deemed a sentinel event in the progression to atherosclerosis (55).

1.2.1 Shear stress

One of the key determinants of the health of the endothelium is shear stress. Defined as the flow-dependent, parallel frictional drag force acting on the vessel wall (56), through forces imposed directly on the endothelium, shear stress can be mechano-transduced into specific biochemical signals that enable it to play a major role in vascular homeostasis (56).

It seems that pulsatile, laminar shear stress stimulates cellular responses that are vital for endothelial cell function. The ability of shear stress to influence endothelial mediated alterations in coagulation, leukocyte and monocyte migration, smooth muscle growth,

lipoprotein uptake and metabolism and endothelial cell survival is strongly atheroprotective (57).

Low wall shear stress is a local risk factor for atherosclerosis. Compared to controls, wall shear stress in the common carotid artery has been shown to be significantly lower in diabetic and hypertensive patients and more recently in patients with symptomatic PAD (58). Furthermore, advanced age, smoking and hypertriglyceridemia are all independent predictors of low wall shear stress (58).

The significance of this becomes clear when one considers evidence demonstrating a strong correlation between ED and areas of low mean shear stress with oscillatory flow associated with flow reversal (57). An example being branch points of the arterial system such as the carotid bifurcation, which are more prone to atherosclerotic lesions than straight segments of vessel.

1.2.2 Endothelial control of vasomotor tone

The endothelium is stimulated by numerous mechanical (eg shear stress) and chemical (eg the neurotransmitter acetylcholine) factors to release a host of vasoactive molecules (*See sections 1.2.2.1* Nitric oxide – synthesis and function *and 1.2.2.3* Endothelin-*1.2.2.4* Prostacyclins, Thromboxane and the Arachidonic Acid pathway*)* ensuring physiological regulation of vasomotor control within the surrounding vascular smooth muscle cells. This is critical for systemic control of blood pressure and to ensure appropriate distribution of blood flow to meet the metabolic demands of different tissues both at rest and during times of increased need induced by stressful stimuli (59). Significantly, many of these molecules play diverse roles and are capable of modulating other endothelium dependent processes such as inflammation and coagulation.

1.2.2.1 Nitric oxide – synthesis and function

One such molecule is nitric oxide (NO). A potent vasodilator critical for the regulation of vasomotor tone, whose pleiotropic effects on the vasculature are many and varied, NO plays an integral role in the homeostatic function of the endothelium (54). Impairment of NO synthesis and bioavailability are regarded as major pathophysiological features of ED (54).

The importance of NO is highlighted by the fact that it was named "Molecule of the year" by *Science* magazine in 1992 and subsequently, research into its function was considered worthy of the Nobel Prize in Physiology and Medicine in 1998. Nitric oxide is a gaseous free-radical produced by the oxidation of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS) (60). There are three isomers of NOS expressed across numerous cell types, demonstrating a role for NO in a variety of biological processes (61): (a) Neuronal NOS – Produces NO in nervous tissue where it has an array of functions including regulation of synaptic plasticity, the sleep wake cycle and hormone secretion (62); (b) Inducible NOS (iNOS) – found in phagocytes, iNOS is activated by interferon-gamma and/or tumour necrosis factor (TNF), NO is secreted as a free radical and is effective in the immune response due to its toxicity to bacteria and other intracellular pathogens (63); (c) Endothelial NOS (eNOS) – Most relevant to this thesis, activity of eNOS requires calmodulin and nicotinamide adenine dinucleotide phosphate as co-factors and a high level of intra-cellular calcium bound to calmodulin (64). It is constitutively expressed but levels of expression and activity can be controlled by biochemical and biomechanical stimuli. Likely acting via a complex secondary messenger system allowing signal transduction, shear stress increases the expression and activation of eNOS and is therefore the most potent and important physiological stimulus for NO production in endothelial cells (64). An increase in vascular shear stress associated with flow related aerobic exercise is one mechanism by which exercise can be protective to the health of the endothelium (65). The sex-hormone estrogen,

neurotransmitter acetylcholine, pro-inflammatory maker TNF-α and products of platelet aggregation serotonin and adenosine diphosphate are also demonstrated activators of eNOS (64).

The structure of eNOS incorporates two subunits. A C-terminal reductase domain and an N-terminal oxygenase domain (64). In the normal state, these subunits are held together by tetrahydrobiopterine (BH_4) in conjunction with heme (Fe). This structure is critical for the reduction of oxygen to NO. In the absence of normal physiological levels of BH_4, the two subunits become "uncoupled" resulting in impaired oxidation of L-arginine and generation of superoxide molecules rather than NO. A futile cycle then ensues as the oxidative damage induced by superoxide molecules and associated reactive oxygen species (ROS), further inhibits BH_4, leading to increased levels of uncoupled eNOS and impairment of NO production that is important to the regulatory activity of the vascular endothelium (64). Given high levels of circulating ROS are associated with cardiovascular risk factors (66) this concept is key to the understanding of how risk factors contribute to low levels of NO and subsequently ED.

Once produced within endothelial cells, NO rapidly diffuses to the neighbouring vascular smooth muscle cells (VSMC's) where it activates the enzyme guanylate cyclase to catalyse the production of cyclic guanosine monophosphate. This in turn activates protein kinase G, which through the phosphorylation of various target proteins causes opening of calcium dependent potassium channels. This leads to hyperpolarisation of the cell membrane, promoting closure of voltage dependent calcium channels, therefore reducing intracellular calcium levels, leading to inhibition of the contractile process (60). The role of NO in vascular homeostasis is not only limited to control of vasomotor tone through interaction and signalling with surrounding VSMC's. After production in the endothelial cells, NO also freely diffuses into the lumen of the blood vessel. Although the half-life of NO in blood has

been estimated in the range of 0.05-1.8 milliseconds, here it has been shown to inhibit platelet adhesion and aggregation, inhibit leukocyte adhesion to the endothelium and inhibit the migration of VSMC's, limiting the neointimal proliferation of such cells in response to injury (67).

1.2.2.2 Asymmetric Dimethylarginine

Asymmetric Dimethylarginine (ADMA) is released by the methylation of arginine residues in proteins by protein arginine methyltransferases and is either renally cleared or metabolised to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (68). Although not leading to "uncoupling", ADMA has been recognised as detrimental to the activity of eNOS and as a result has been deemed a novel marker of ED (68, 69). It is an endogenous analogue of the eNOS substrate L-arginine and is therefore a competitive inhibitor of L-arginine. A balanced L-arginine:ADMA ratio is critical to ensure optimal eNOS activity (69). Oxidative stress has been shown to inhibit DDAH activity, resulting in increased levels of ADMA, a reduction of NO synthesis and ED (68, 69). Given the established link between oxidative stress and cardiovascular risk factors, it is not surprising that numerous studies have reported high levels of ADMA in association with diabetes mellitus (70), hypertension (71) and hypercholesterolemia (72). Thus, the regulation of ADMA provides insight into the proposed link between CVD risk factors and ED. Providing support to such a link is the study from Boger et al(72) who demonstrated normalisation of endothelial function after administration of intra-venous L-arginine to hypercholesterolemic patients with elevated ADMA levels. Given that raised ADMA levels can modify the effectiveness of statin therapy, a potential therapeutic target for the future may be to combine the effects of L-arginine administration and statin therapy (73).

1.2.2.3 Endothelin

Also contributing to the endothelial control of vasomotor tone, Endothelins are a family of 21 amino acid peptides, of which endothelin-1 is produced by endothelial cells (74). Although constitutively produced, some regulation is provided by inflammatory markers such as transforming growth factor-β (TGF-β) and TNF-α. Once released, endothelin-1 preferentially binds to endothelin-a receptors on VSMC's. Activation of these G-protein coupled receptors leads to an increase in intracellular calcium and ultimately vasoconstriction. The most potent of vasoconstrictors, in a healthy endothelium, the effects of endothelin balance the vasodilatory activity of NO. Reduced levels of NO in ED tilt the balance in favour of the vasoconstrictive effect of endothelin.

1.2.2.4 Prostacyclins, Thromboxane and the Arachidonic Acid pathway

Another important contributor to vasomotor tone, whose release from endothelial cells is stimulated by shear stress, is prostacyclin. A member of the family of lipid molecules known as eicosanoids, prostacyclin is metabolised in endothelial cells from endogenous arachadonic acid through the cyclo-oxygenase pathway. This pathway begins with cell membrane phospholipids being acted upon by the enzyme phospholipase A and converted to arachidonic acid. This is then converted to the intermediate compound cyclo-endoperoxides by cyclo-oxygenase enzymes. Tissue specific enzymes then convert cyclo-endoperoxides into biologically active eicosanoids. Prostacyclin, produced by endothelial cells is a potent vasodilator (75) but also an inhibitor of platelet aggregation (57). In contrast, thromboxane, an eicosanoid produced by platelets, is a potent vasoconstrictor whose production is enhanced by inflammation and tissue injury and following platelet activation. The right balance between prostacyclin and thromboxane is therefore important to facilitate endothelial control of vasomotor tone and thrombosis.

1.2.3 Endothelial Control of Thrombosis and Coagulation

Central to the control of haemostasis and thrombosis (the ability of blood to clot), the endothelium not only provides a protective layer separating blood from the pro-thrombogenic vessel wall, but also serves as the primary source of major haemostatic regulatory molecules. The properties of a healthy endothelium therefore ensure that an anti-thrombotic and anti-coagulant balance are maintained within the blood stream.

Platelets are small, anuclear cells, that play an integral role throughout the process of haemostasis and thrombosis. The ability of the endothelium to regulate their activity is therefore vital and the importance of the contrasting effects on platelets of thromboxane and prostacyclin as well as the ability of NO to act as an inhibitor of platelet aggregation have been discussed in *sections 1.2.2.4* Prostacyclins, Thromboxane and the Arachidonic Acid pathway *and 1.2.2.1* Nitric oxide – synthesis and function respectively. Numerous other mechanisms also exist.

1.2.3.1 Thrombin

The most potent activator of platelet activity is an enzyme called thrombin. A serine protease, thrombin is also known as clotting factor II and can also activate numerous other clotting factors including factors V, VIII and XI. In addition, thrombin facilitates the cleavage of fibrinogen to fibrin which stabilises clot formation and further stimulates platelet aggregation. (76)

The healthy endothelium is capable of inhibiting thrombin activity through the expression of anti-thrombotic haparan sulphate proteoglycan molecules to line the cell surface. These molecules allow binding and a subsequent conformational change of anti-thrombin-III, an inhibitor of thrombin, therefore facilitating the inactivation of thrombin. (77). Such inhibition is also augmented by shear stress which is known to stimulate secretion of haparan sulphate.

Another anti-thrombotic strategy employed by the endothelium is the expression of Tissue Factor Pathway Inhibitor. This binds to and inhibits clotting factor Xa which is required for the activation of thrombin from pro-thrombin, thereby reducing the levels of bioactive thrombin (51, 78).

1.2.3.2 Thrombin-Thrombomodulin Complex

Like haparan sulphate, thrombomodulin is a membrane protein expressed on the surface of endothelial cells. It acts as a co-factor for thrombin and when the pair are in complex, the pro-coagulant properties of thrombin are inhibited and the anticoagulant Protein C is activated (76). Activated Protein C and its co-factor Protein S are both vitamin-K dependent and inactivate clotting factors Va and VIIa.

In balance with this, the Thrombin-Thrombomodulin Complex also has pro-coagulant properties by activating Thrombin Activatable Fibrinolysis Inhibitor, resulting in a loss of fibrinoltyic enzymes such as plasmin and therefore inhibiting fibrinolysis.

1.2.3.3 Coagulation in a dysfunctional endothelium

While the phenotype of a healthy endothelium is, on-balance, anti-coagulant, one of the defining features of a dysfunctional endothelium is a pro-coagulant, pro-thrombotic phenotype. The anticoagulant mechanisms described above (*See sections 1.2.3.1* Thrombin-*1.2.3.2* Thrombin-Thrombomodulin Complex*)* are impaired and the dysfunctional endothelium is characterised by the expression of tissue factor (TF), which is not detected on the normal endothelium. It is widely recognised that TF forms a complex with factor VIIa, to initiate both the extrinsic and intrinsic coagulation cascades (79).

1.2.4 Inflammation

Just as the phenotype of a healthy endothelium is anti-thrombotic and anti-coagulant, it is also anti-inflammatory and endothelial cells play an important role in the initiation and

regulation of inflammatory and immune responses (51, 52). Inflammation can be considered as a response to endothelial cell activation (80).

Endothelial cell activation is divided into two stages and occurs in response to stimuli such as changes in shear stress or oxidative stress, hypoxia, toxins, hyperlipidemia and other risk factors for CVD (81).

1.2.4.1 Type I Endothelial Cell Activation

Type I endothelial cell activation occurs immediately following stimulation and is mediated by ligands (eg histamine) of G-protein coupled receptors. The subsequent rise in cytosolic calcium concentration and formation of a calcium-calmodulin complex leads to phosphorylation of myosin light chains and facilitates contraction of endothelial cells. The associated loss of contact between adjacent endothelial cells increases vascular permeability and can cause haemorrhage and oedema (82).

An increase in cytosolic calcium levels in endothelial cells also plays a major role in leukocyte adhesion and migration and the subsequent extravasation of inflammatory cells from the vessel lumen to the tissue interstitium.

Through exocystosis and an increased display of proteins P-selectin and Platelet Activating Factor on the endothelial cell surface membrane, juxtacrine signalling from activated endothelial cells allows leukocytes to begin “rolling” along the endothelium. This occurs via the formation of transient bonds mediated by a family of proteins called selectins which are expressed on leukocytes (L-selectin), endothelial cells (E-selectin and P-selectin) and platelets (P-selectin) and their respective ligands (83). These low-affinity bonds slow the leukocytes allowing them to ultimately become immobilised and tightly adherent to the endothelium. This process is mediated by a family of leukocyte surface proteins referred to as

integrins, which bind to their ligands on the endothelial surface, typically vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) (83).

Once leukocytes are firmly adherent to the endothelium, they can begin the process of migration across the endothelium and vessel wall. This typically occurs through intercellular junctions between endothelial cells and is facilitated by several additional adhesion molecules present in the junctions including platelet endothelial cell adhesion molecule (PECAM). The leukocytes then secrete proteolytic enzymes enabling them to cross the basement membrane of the endothelial cells and enter the extravascular space, where they are drawn towards their desired location (generally the site of injury) by a process known as chemotaxis. This involves leukocyte recognition of chemical signals produced by various exogenous and endogenous molecules called chemoattractants and movement of leukocytes towards these signals (83).

1.2.4.2 Type II Endothelial Cell Activation

Type II endothelial cell activation is a delayed response in which inflammatory cytokines (TNF or Interleukin-1) derived from activated leukocytes stimulate activation of gene expression and de novo protein synthesis (84).

Cytokines are a family of short-acting proteins that are known as the messenger molecules of the immune system. They are produced by various cell types and are crucial for cell signalling. There are various types of these immunoregulatory proteins, often named according to their secretory or target cell (eg interleukins because they predominantly target and mediate interactions between leukocytes) (85).

Due to the synthesis and display of new chemokines and cellular adhesion molecules (CAM's) such as E-selectin, leukocyte adhesion and migration (S*ee section 1.2.4.1* Type I Endothelial Cell Activation*)* is more effective following type II endothelial cell activation

rather than type I activation. This also allows for a more sustained response, capable of evolving over time, such that the time-dependent change in inflammation from neutrophil dominated to mononuclear cell responses can be attributed to changes in molecules expressed by type II activated endothelial cells (80).

1.2.4.3 Chronic Endothelial Cell Activation and Endothelial Dysfunction

A healthy endothelium is responsible for maintaining vascular homeostasis, however, uncontrolled, chronic and persistent endothelial cell activation such as exposure to modified lipoproteins, increased levels of oxidative stress and haemodynamic insults associated with CV risk factors can result in irreversible endothelial cell injury and endothelial dysfunction.

The result is a reduction in NO bioavailability, greater expression of tissue factor and an increase in secretion of pro-inflammatory cytokines and therefore increased expression of CAM's with a subsequent increase in leukocyte adherence and migration. As a consequence of these changes, the phenotype of the endothelium becomes vasoconstrictive, pro-inflammatory, pro-thrombotic and hypercoagulant (54).

The extent to which the phenotype favours endothelial dysfunction can be determined by the balance between exposure to risk factors and the reparative capacity of endothelial cells. Adjacent endothelial cells can replicate locally and replace lost or damaged cells. More importantly, circulating stem cells known as endothelial progenitor cells are recruited from the bone marrow to peripheral blood and can differentiate into mature cells with endothelial properties. Demonstrating the importance of such cells, recent work has shown that participants with a greater circulatory number of endothelial progenitor cells have preserved endothelial function despite exposure to high levels of cardiovascular risk factors (81).

1.2.4.4 Endothelial Dysfunction and Atherosclerosis

The concept that atherosclerosis is a chronic inflammatory disease initiated by leukocyte adhesion to dysfunctional endothelial cells is widely accepted (86) and is a platform on which to further explain the link between ED and progression to established disease. After migration into the extravascular space, the subset of leukocytes called monocytes (bipotent cells produced in the bone marrow) differentiate into phagocytic cells known as macrophages (87). The uptake of oxidised low-density lipoproteins (LDL's) by these macrophages leads to the formation of foam cells, which, through the secretion of cytokines, can further precipitate inflammatory cell recruitment (87). The aggregation of lipid rich macrophages and other inflammatory cells forms a fatty streak, the earliest lesion in the onset of atherosclerosis. The fatty streak then becomes a mature atheroma after growth factors expressed from adherent leukocytes and platelets facilitate the recruitment and proliferation of smooth muscle cells (SMC's) from the circulation. These SMC's synthesise collagen, elastic fibres and proteoglycans to form an extracellular matrix, which further modifies the atheroma, typically forming a fibrous cap to surround a core of lipid laden inflammatory cells. In time, these atheromas become calcified and stable, or the fibrous cap can rupture leading to thrombosis and embolism which characterise acute cardiovascular events (83).

Significantly, not only does a healthy endothelium prevent leukocyte adhesion and migration, but the secretion of a "normal" level of NO is capable of inhibiting the oxidation of LDL's and proliferation of SMC's (59). The reduced bioavailability of NO in association with ED means that the impact of NO is outweighed by the presence of vasoconstrictors such as endothelin, which the dysfunctional endothelium continues to secrete in normal amounts (59). Both of these molecules are also pro-oxidant and pro-proliferative and therefore pro-atherogenic, consistent with the theme of ED.

Dysfunctional endothelial cells may also play a role in tissue fibrosis and subsequently progression of atherosclerosis, via a recently identified concept known as endothelial-mesenchymal transition. In the setting of chronic inflammation, as is the case with the pro-inflammatory state of ED and atherosclerosis, exposure to inflammatory cytokines TGF-β, TNF-α and Interleukin-1B, can facilitate the differentiation and transformation of endothelial cells to fibroblasts (mesenchymal cells). In response to further pro-inflammatory signals, these fibroblasts become activated and increase secretion of extra-cellular matrix which contributes to tissue remodelling and fibrosis (88).

1.2.5 Assessment of endothelial function

Endothelial dysfunction has been established as an important early event in the pathogenesis of atherosclerosis with changes in endothelial function occurring well before the onset of clinically apparent CVD (53, 54). Furthermore, ED has been shown to predict cardiovascular events and to correlate with known CVD risk factors (54). With this in mind, techniques have been developed to measure endothelial function allowing for risk stratification and prediction of the likelihood of future CVD events, but also the assessment of disease severity and monitoring of the efficacy of risk modification therapies.

Many non-invasive methods for measuring endothelial function have been investigated with two of the most commonly used being flow mediated dilatation (FMD) and peripheral artery tonometry (PAT).

FMD is the most established and commonly used non-invasive method for assessing endothelial function. The FMD technique is based on the reactive hyperaemia phenomenon of increased arterial blood flow following a period of transient arterial occlusion (53, 89)}. This increase in blood flow results in an increase in shear stress on the vessel wall which induces the release of nitric oxide (NO) by endothelial cells, causing vasodilatation. This

response is termed NO-mediated flow mediated dilatation and is typically measured in the brachial artery (89). It has become apparent that the position of the occlusive cuff has a significant effect on FMD (53). Proximal, upper arm, occlusion causes a greater dilatation but this is not solely a NO-mediated response as it also measures the hyperemic response to ischaemia-induced hypoxia. Distal, forearm occlusion causes a lesser, but more specifically NO-mediated dilatation and has recently become the recommended FMD method (53).

Peripheral arterial tonometry is a more recently developed method that has been used to measure endothelial function. Peripheral arterial tonometry uses pneumatic finger probes to measure digital arterial pulse wave amplitude when reactive hyperaemia is induced. Ease of use, standardised methodology and the availability of validated cut-off thresholds to predict future cardiovascular events have encouraged the use of PAT in studying endothelial function.

The degree to which the two tests correlate with one another is not clear and the use of different occlusion methods for FMD has confused the available evidence, with most studies investigating correlation using proximal occlusion FMD (90-93) and fewer studies using the currently recommended technique of distal occlusion (94-96).

1.3 Exercise Training

1.3.1 Benefits of exercise

There is mounting evidence to support the physical and psychological benefits of regular physical activity in men and women of all ages. An energy expenditure of approximately 1000 kcal/week through moderate intensity physical activity has been demonstrated to lower the rate of CVD and premature mortality (97). Treadmill testing has demonstrated that middle aged adults with moderate compared to low levels of cardio-respiratory fitness have a 60%

lower rate of all cause and cardiovascular related mortality (97). The reasons for these findings are likely to be multi-factorial. Psychologically, regular exercise leads to feelings of enhanced energy, well-being and quality of life and helps to prevent a decline in cognitive function and a state of depression and anxiety (97). Such factors have previously been implicated in the maintenance of physical health. More obviously, from a physical perspective, improvement in body composition (particularly skeletal muscle mass and quality) leads to a reduction of the number of pro-inflammatory adipocytes, while other cardiovascular risk factors (blood pressure, lipoprotein profile, insulin sensitivity) are also significantly modified (97). Ultimately, systemic inflammatory burden may be reduced, having an associated positive impact on individual health outcomes.

1.3.1.1 Exercise and the endothelium

To gain a more thorough understanding of the influence and benefits of regular exercise on long-term health outcomes, it is necessary to consider the impact of exercise on endothelial function. The health of the endothelium has already been implicated in the pathogenesis of atherosclerosis and PAD through its control of vascular homeostasis, however, it also plays a vital role in modulating the health benefits induced by exercise. There are several mechanisms accounting for the improvement of endothelial function induced by exercise. Perhaps the largest contribution, particularly in healthy individuals, comes from the augmentation of blood flow and shear stress, resulting in increased endothelial NO production and up-regulation of eNOS activity (the same mechanism causes FMD) (65, 98). As has already been discussed *(See section 1.2.2.1* Nitric oxide – synthesis and function), bioavailability of NO is critical to achieve the desirable homeostatic outcomes of the endothelium and is a surrogate marker for endothelial function. Further improvement in endothelial function through physical activity may be achieved as a result of inhibition of the biological ageing profile of cells in the vessel wall and subsequent prevention of apoptosis

(99). Similarly, an increased level of stem cells capable of inducing endothelial repair and angiogensis (endothelial progenitor cells) has been demonstrated (100). It is likely that an improvement in the cardiovascular risk factor profile also plays a key role. In particular, improved insulin sensitivity and lipid status results in a reduction in oxidative free-radicals which are capable of activating the normally quiescent endothelium into a dysfunctional state through the inhibition of NOS (101). Furthermore, altered body composition and a lesser volume of adipocytes also has an anti-oxidative and anti-inflammatory effect (97). Combined, greater endothelial function will result in an improved anti-inflammatory, anti-thrombogenic phenotype of the endothelium and helps to explain the beneficial effects of exercise on health outcomes.

To confirm the beneficial effects of exercise on the endothelium, several studies of varied patient cohorts have used FMD as a surrogate marker of endothelial function. In a cohort of young, healthy male, military recruits, Clarkson et al (98) demonstrated a significant improvement in FMD after a 10 week regimen, consisting of aerobic and anaerobic exercise, was undertaken. Such findings were confirmed by Kasikcioglu et al (102) who, based on their findings that FMD was significantly higher in athletes (n=32, FMD=17.1%) than controls (n=30, FMD=11.2%), concluded that habitual aerobic exercise imparts better vascular adaptation than a sedentary lifestyle. This is an important concept to consider in older adults, in whom physiological and sometimes pathophysiological changes lead to the deterioration of markers of vascular health and function (arterial wall stiffness, endothelial function) and contribute to an increased cardiovascular risk with ageing (103). Providing evidence to suggest that the potential for beneficial vascular adaptation is not confined to younger individuals, DeVan et al (103) undertook a review to assess vascular health in middle aged and older athletes. They suggested that in those older adults who undertook solely endurance based training, FMD was better than older untrained athletes, although not as good as

younger untrained individuals. The findings however, were quite different for those older adults undertaking resistance training. Such training was found to negatively impact on markers of arterial function such that they were worse than baseline measures of untrained older adults, thus placing such a cohort of individuals at a higher cardiovascular risk than would otherwise be the case. While a precise explanation for such a finding is lacking, it has been speculated that differences in intravascular forces generated by resistance training may be accountable (103). This is consistent with findings that weight lifting can induce large, transient increases in arterial pressure with an associated pro-inflammatory and pro-oxidant response detrimental to endothelial function (104). High intensity exercise has also been shown to be detrimental to vascular health due to the associated high levels of oxidative metabolism and pro-oxidant environment (105). In contrast, the anti-oxidant benefits associated with moderate intensity exercise are crucial for positive adaptations and preservation of vascular function (105). Such findings highlight the clinical importance of appropriate prescription of exercise regimens, with respect to intensity, duration, frequency and modality.

1.3.1.1.1 Sex-specific differences

Consideration should also be given to potential sex-specific differences in endothelial response to exercise. In a cross-sectional study (n=211), Pierce et al (106) demonstrated a significantly better FMD in endurance trained older men compared with age-matched sedentary men (6.4% vs 4.3%, P=0.001). This finding did not extrapolate to post-menopausal women, who demonstrated no difference in FMD among endurance-trained and sedentary individuals (5.3% vs 5.6%). In support of this finding, sedentary participants from the same patient cohort were subjected to an eight week treadmill based exercise intervention. Sedentary male participants demonstrated improvements in FMD (4.6% to 7.1%, P<0.01) while post-menopausal women did not (5.1% to 5.4%, P=0.50). Conflicting results are

presented by Black et al (107), who, in a small cohort of volunteers (n=16, 8M, 8W), suggested that a 12 week exercise intervention improved vascular function in sedentary older women (post-menopausal), but not in men. Such conclusions were based on an FMD:Glyceryltrinitrate (GTN) ratio, to determine NO dilator function in the context of smooth muscle cell sensitivity, where FMD characterises endothelium dependent vasodilatation and GTN administration is used to assess endothelium independent vasodilation. Of note, while the FMD:GTN ratio was significantly improved following exercise in sedentary older women, when taken independently, neither FMD or GTN had changed significantly (mean values not stated). Results from this study should therefore be interpreted with caution.

While further evidence is undoubtedly required, it has been speculated that the low levels of estrogen present in post-menopausal women maybe insufficient to enable the modulation of vascular endothelial function in response to exercise (103). If proven to be correct, this may have ramifications for the interpretation of the efficacy of all exercise interventions proposed to be undertaken by older women, particularly when using FMD as an outcome measure.

1.3.1.1.2 Patients with co-morbidities

Despite these findings, it is still clear that for the most part, when prescribed appropriately, exercise has a beneficial effect on the endothelium in healthy individuals. There is also overwhelming evidence of the influence of exercise on the endothelium in those with underlying health conditions, often with subsequent improvement of the condition and associated prognosis.

In a randomised controlled study of 26 patients with long-standing yet uncomplicated type-1 diabetes (mean age 42 years, 12M), those who undertook a 16 week supervised cycle based exercise intervention were shown to have significant improvements in their FMD compared

with controls (change in FMD 3.2% vs -0.7%, P<0.05) (108). Importantly, this study is one of the first to also assess the impact of exercise cessation on long-term vascular function and suggested that all benefit of exercise on the endothelium is lost within eight months following cessation.

Finally, the ultimate endorsement for the beneficial effects of exercise on the endothelium is given by Hambrecht et al (109), who in a publication in the *New England Journal of Medicine* used coronary angiography and acetylcholine challenge (the gold standard of assessment of endothelial function) to demonstrate that in patients with established CAD, a four week cycle-based supervised exercise program was capable of significantly improving endothelium dependent vasodilatory responses within the coronary vasculature (change in the %change in mean peak coronary blood flow velocity after administration of acetylcholine following four weeks of exercise 15.2% vs 1.2% in non-exercising controls, P=0.001).

1.3.2 Exercise training in patients with Peripheral Arterial Disease

Based on the above discussion, it seems logical to conclude that exercise in patients with PAD should improve endothelial function and the underlying cardiovascular risk factor profile, thus providing significant benefit in minimising disease progression and reducing the high risk of cardiovascular morbidity that is associated with the condition. Indeed, there is also a growing body of evidence to support a role for exercise in providing symptomatic improvement for patients with PAD, in particular IC (110, 111). The consensus guidelines published on PAD are consistent with these reviews, with the authors suggesting that exercise should be made available as part of the initial treatment for all patients with PAD (7, 26). Furthermore, the guidelines suggest that the most effective exercise regimens are supervised and involve walking based programs of at least one hour two-three times per week, of sufficient intensity to induce claudication.

Despite these recommendations, the role of walking based exercise alone in the treatment of IC has not actually been independently assessed. Recent reviews (111) and meta-analyses (110) have considered a range of exercise modalities, often in combination, including walking, dynamic leg exercises and upper and lower limb resistance training. In fact, relatively few articles have considered the impact of walking alone on functional performance in patients with intermittent claudication.

A review of this topic was therefore performed by electronically searching the literature published from inception of various databases to July 2014. Data sources included Medline, US National Library of Medicine (PubMed), Scopus, Cochrane Collaboration Library and Google Scholar. Bibliographies of all eligible papers were manually searched. The search terms used to identify relevant studies were: peripheral arterial disease, peripheral vascular disease, intermittent claudication, supervised exercise and treadmill. All studies for inclusion were required to meet the following criteria: (a) Randomised controlled trial (RCT) published in the English language in which a supervised, treadmill-based exercise regimen only (as suggested by the TASC II consensus recommendations) was compared to a usual care control (ie managed with "best medical therapy"). (b) Inclusion of participants with claudication only (Rutherfords stage I-III classified disease) and not CLI. (c) Utilisation of a walking assessment tool to determine time or distance to onset of claudication pain [pain free walking time (PFWT) or pain free walking distance (PFWD)] and the point at which further walking was limited by intolerable claudication pain [maximum walking time (MWT) or maximum walking distance (MWD)]. (d) Duration of intervention no less than 4 weeks.

1.3.2.1 Walking performance

There were 18 suitable studies included incorporating 810 participants, whose study design involved randomisation and a direct comparison of a treadmill based walking intervention to a usual care control group. Of these, 12/18 (49, 112-122) reported statistically significant

improvements in at least one marker of walking performance compared with control groups, a further 5/18 (123-127) demonstrated a statistically significant improvement from baseline to post-intervention, of which 3/5 (123, 124, 127) studies did not mention a comparison with the control group despite being referred to as RCT's. One study (128) showed a non-significant reduction in walking distance after the intervention, likely due to the observed variability in response to treadmill-training.

Throughout these trials, marked variation exists between the values reported for primary outcome measures. Maximum walking time and pain-free walking time are the measures most commonly referred to and at baseline, the range of means reported is 5.3 (49) to 16 minutes (116) and 1.6 (49) to 7 minutes (116) respectively. Following intervention, the range of mean change reported for MWT is 3.6 (120) to 26 minutes (123) versus -0.2 (116) to 1.2 minutes (112) in control groups and for PFWT 2.9 (115) to 27.2 minutes (123) versus -0.7 (123) to 1.3 minutes (125) in control groups. Similarly, for those trials that report MWD and PFWD, baseline mean values range from 258m (128) to 484m (121) and 87m (127) to 200m (121) respectively with the mean change in MWD following intervention ranging from -11m (128) to 360m (113) versus -9m (128) to 46m (114) in control groups and for PFWD 92m (121) to 250m (113) versus 4m (121) to 40m (114) in the control groups. While the heterogenous nature of the studies may account for some variability, it is interesting to note that Murphy et al (49) alone reported a range of change in MWT from -0.4 to 16.8 minutes, suggesting that the ability of an individual to respond favourably to exercise training may be multi-factorial, including genetic and lifestyle factors. The ability to predict which patients are likely to respond favourably to exercise is therefore an important consideration. The following discussion may enable some understanding of factors that can potentially predict outcomes of supervised, treadmill-based exercise training in patients with IC.

1.3.2.2 Heterogeneity of studies

1.3.2.2.1 Participant demographics

The demographics of patients within each trial suggest that PAD is more prevalent in the older, male population and particularly among patients with underlying cardiovascular risk factors. Such demographics were generally well matched between groups indicating that randomisation had been performed successfully. Smokers were excluded in early studies from Gardner et al (113, 114), however, the use of MWD and PFWD rather than the more commonly used MWT and PFWT makes comparison with other studies difficult. Diabetics were excluded in four studies (112, 123, 124, 127) based on the premise that poor glycaemic control may affect the response to an exercise program. Interestingly, the two studies from Hiatt et al, which excluded diabetics, (112, 123) led to the greatest reported improvement in MWT of all studies, albeit, each study consisted of only 10 subjects per group, such that in one study, statistical significance was not reached. Mika et al (127) and Regensteiner et al (124), who also excluded diabetics, both reported improvements in walking performance following exercise, but these were not significant compared to control groups. It is therefore difficult to draw meaningful conclusions with respect to the impact of diabetes on response to exercise in patients with IC.

1.3.2.2.2 Anatomical location of disease

Anatomical distribution of disease as well as disease severity (subjectively reported by participant as PFWD and MWD and objectively obtained through ABPI's) may impact on the ability of a patient with PAD to respond to exercise.

Patients with aorto-iliac disease are likely to have a greater surface area of ischaemic muscle tissue than those with infra-inguinal disease, potentially reducing the impact of an exercise intervention. While in most identified studies, the anatomical location of disease is not stated,

Murphy et al (49) only included patients with evidence of haemodynamically significant disease in the aorto-iliac segment. Previous work has demonstrated that patients with aorto-iliac disease are more symptomatic than those with distal disease (129). Consistent with this, among the studies that used the same method of assessment, the 43 patients in the treadmill based exercise group from Murphy et al (49) were found to perform the worst on baseline assessment of walking performance, with PFWT of 1.6 minutes and MWT 5.3 minutes. Similarly, the mean three minute improvement of PFWT in these patients, although statistically significant, was at the lower end of the spectrum and therefore of questionable clinical meaningfulness. While the results from Murphy et al (49) may be weakly suggestive that patients with aorto-iliac disease respond less favourably to a treadmill-based exercise program, without any documentation of anatomical location of disease in the other 17 studies, no meaningful conclusion can be made about the role location of disease plays in predicting response to exercise.

1.3.2.2.3 Disease severity

The concept of disease severity is an interesting one, comprising of both subjective and objective measures, both of which are in some way flawed. Objectively, ABPI's were used in each study to quantify lower-limb blood flow and confirm a diagnosis of PAD. In most cases, patients were eligible for inclusion if their resting ABPI was <0.90, although Gelin et al (128) required a value of <0.60 and Gibellini et al (119) included participants with ABPI <0.70. Several studies also required a baseline post-exercise reduction in ABPI of >0.20 (116, 123-125). Mean baseline resting ABPI ranged from 0.54 (128) to 0.72 (117, 118). Of note, Gelin et al (128) had the largest cohort of patients (n=73 exercise, n=76 control) and was the only group to record a non-significant reduction in MWD, raising the possibility that a lower resting ABPI is associated with a poor response to treadmill training. Two other studies had mean baseline resting ABPI values <0.60 (123, 124) and failed to demonstrate a significant

improvement in walking performance following an exercise intervention, as compared to usual care controls. Given a significant improvement within group from pre to post exercise, this is likely reflective of a type 2 error arising from the small number of subjects in these trials. In fact, Hiatt et al (123) actually demonstrated the largest within group improvement in mean MWT (9.6mins to 14.7mins, $p<0.05$). Again, like most other potential predictors of response to exercise, the lack of sub-analyses within published literature and the absence of studies specifically dedicated to investigate such a research question means that reaching meaningful conclusions is challenging or not possible.

While ABPI has been well established as a marker of disease severity, it is interesting that in four studies (49, 123, 124, 128) to report ABPI's post intervention, there was no significant change from baseline values, despite significant improvements in walking performance. While this is likely to reflect the underlying mechanisms by which exercise has been proposed to positively benefit walking performance (*See section 1.3.3* Mechanism of response to exercise), it brings into question the validity of such a measure in assessing response to an exercise intervention.

The subjective alternative in the assessment of disease severity is pain free walking performance and is used as the primary outcome measure in each trial, the ranges of which have been detailed above. There was no obvious trend detectable between those studies reporting extremes of baseline pain free walking times/distances and the extent of improvement following exercise.

Based on the available evidence, it is therefore difficult to account for the marked variability in walking performance observed across the trials, using patient and disease characteristics. Despite this, it remains highly likely that these and other genetic based factors play a role in determining response to exercise.

1.3.2.2.4 Exercise intervention

The heterogeneity of the exercise interventions undertaken in these studies may also influence the observed variability of response to treadmill based exercise training. The duration of intervention ranged from 4 weeks (119) to 12 months (117, 128) and exercise protocols were many and varied. Patients were asked to attend three sessions per week of intermittent treadmill training, with the exception of Hodges et al (126) who required two sessions per week, consisting of several cycles of walking to various extremes of claudication pain, rest until resolution of pain before recommencing walking. This tended to be in a progressive manner with an initial speed of 3.2km/hr (2mph) and a gradient of 0°, increasing as determined by the walking performance of the participant. Some studies specified the amount of actual walking time required within each session (113-118, 120, 122, 125, 128) while others were non-specific in stating the duration of the session as a whole (49, 112, 121, 123, 124, 126, 127). There were however, some interesting variations in protocol with respect to intensity and duration of the intervention. Both Wood et al (125) and Sanderson et al (116) used 6 week relatively high intensity, interval treadmill training which required participants to walk at 3 sessions per week for 10 x 2 minute intervals, each separated by 2 minutes of rest. For the first 3 weeks, intensity was 80% of VO2 max (the maximal rate of oxygen consumption of an individual) determined by initial incremental treadmill testing, increasing to 100% for the second 3 weeks of the program. Using such a protocol, Sanderson et al (116) reported significant improvements in both PFWT and MWT compared with usual care controls (PFWT 412 to 607secs versus 391 to 446 secs, $P<0.05$ and MWT mean difference 240 secs versus -10secs, $P<0.05$). This was in contrast to Wood et al (125) who did not demonstrate group differences, the very small sample size (n=7 per group) potentially masking any potential significance. Further insight into the role of walking intensity has been provided by Gardner et al (130) who conducted a 6 month intervention, randomising patients

to either low-intensity exercise (40% of the maximal workload achieved during baseline treadmill test) or high-intensity exercise (80% maximal workload with reduced volume of exercise to control for caloric expenditure). While each group demonstrated significantly improved PFWD and MWD, there was no statistical difference between groups, suggesting that the intensity of exercise may not be a critical factor in dictating response to exercise.

The 6 week duration of exercise intervention in both Wood et al (125) and Sanderson et al (116) was also relatively short compared to most other identified studies whose intervention typically ranged from 3-12 months. The benefit of a relatively short exercise program was further explored by Gibellini et al (119), who randomised 20 patients with IC to a 4 week treadmill training program (30mins relatively low intensity treadmill training twice/day, 5 days/week) and 20 patients with IC to a usual care control group. Treadmill training resulted in significant improvements in both PFWD and MWD (PFWD 109m to 251m versus 111m to 111m, $P<0.05$ and MWD 217m to 450m versus 230m to 226m, $P<0.05$). Whether or not exercise beyond the 4 week intervention would have resulted in yet a greater improvement in walking performance was not assessed by Gibellini et al (119). Such a concept was however, considered by three other independent research groups, with mixed results. In a 6 month moderate intensity graded treadmill intervention, Treat-Jacobsen et al (121) demonstrated no improvement in walking performance (measured as PFWD and MWD either within or between groups) from 3 months to 6 months. This finding was supported by Hodges et al (126) whose participants undertook two x 1hour sessions/week of intermittent treadmill training and improved their walking performance in the first 6 weeks but then failed to demonstrate further improvement from 6 weeks to 3 months. In contrast, using very similar exercise protocols (three x 1hour sessions/week of intermittent treadmill training), Hiatt et al (123) demonstrated significant improvement in walking performance from 3 months to 6 months (PFWT 14.7mins to 17.2mins, $P<0.05$).

Gardner et al (113) took a slightly different approach to exploring this concept. Following a 6 month progressive treadmill based intervention, participants then engaged in a 12 month maintenance treadmill program to determine the sustainability of the initial significant improvement in walking performance. Although no further improvement was demonstrated after maintenance exercise, performance was not significantly reduced at this point. Gibellini et al (119) also showed that short-term improvements in walking performance could be maintained without maintenance exercise. Six months after 4 weeks of supervised treadmill exercise, Gibellini et al (49) demonstrated that the initial significant improvement in PFWD and MWD had been maintained. Although the exact mechanism behind such sustained improvement is unclear, it likely reflects the motivation of participants to continue with physical activity at home. Regardless, the evidence certainly supports a lasting improvement in walking performance following a treadmill based intervention.

The above issue of self-prescribed home-based exercise training is worth mentioning as it was poorly controlled in the majority of studies. In two studies (116, 120), controls were advised to self-prescribe exercise as part of usual medical care protocols but no additional advice was given to those enrolled in supervised programs. A further four studies (49, 121, 125, 126) provided advice to both control and exercise groups, while in another study (128), controls were neglected while the exercise group were advised to self-prescribe exercise in addition to supervised exercise. The remaining 10/18 groups did not provide any advice with respect to exercise outside of the intervention. The significance of this is highlighted by the only group whose protocol required participants to provide diary records of physical activity engaged in throughout the program (121). Interestingly, despite providing the same advice to both groups, diary records suggested that 45% of participants in the exercise group engaged in an additional 2 or more self-prescribed sessions per week, compared with 75% of controls who participated in 3 or more sessions per week. While this did not prevent Treat-Jacobsen et

al (121) from demonstrating significant improvement in walking performance, it certainly highlights a confounding factor that has not appropriately been accounted for in most studies. Other studies have provided information on physical activity levels through the use of accelerometers (122), pedometers (49) and double labelled water (114), however, these have been used as secondary outcome measures performed before and after intervention in an attempt to determine changes as a result of exercise, rather than monitoring activity levels during the intervention.

1.3.2.2.5 Primary outcome assessment

Critical to the success of scientific literature is the primary outcome assessment and the method by which it is obtained. As has been mentioned extensively above, in the case of exercise interventions for IC patients, walking performance, judged essentially by claudication distance is the primary outcome. In the words of Watson et al (131) however, "claudication distance is spuriously estimated, inaccurately reported, falsely recorded, inappropriately measured and usually misinterpreted". Despite this, it is the basis on which Level A evidence for the treatment of IC has been derived.

1.3.2.2.6 Terminology

To put this statement into context, it is necessary to start simply with a brief discussion on the need for standardisation of the method of assessment for walking performance in IC. Perhaps the crux of the problem is highlighted quite simply by the variation that exists in terminology and parameters reported from treadmill testing. As was mentioned earlier, either units of time or distance are used with respect to pain free or maximum walking ability. Walking distance however, seems a more meaningful clinical measure of functional performance than walking time.

In addition to the issue of time versus distance, is the variability of terminology used to express either pain free or maximum walking ability. For the purposes of this review, such terminology has already been converted, but terms such as claudication onset time (COT) (49) or initial claudication distance (ICD) (119) were used to report pain free walking ability, while peak walking time (PWT) (49) and absolute claudication distance (ACD) (119) were equivalent to maximum walking ability. To avoid misinterpretation of such terminology, Duprez et al (132) has recommended that in future literature the use of terminology should be standardised and only one term used. Interestingly, in a recent study, Kruidenier et al (133) investigated the validity of a new term, functional claudication distance (FCD). Defined as the distance at which a patient prefers to stop, rather than the maximum or pain free walking distance, they demonstrated FCD to be a reliable and valid measurement for determining functional capacity in patients with IC. Furthermore, it was proposed to better reflect the actual functional impairment in these patients and has been suggested as an alternate parameter in the assessment of such patients.

1.3.2.2.7 Method of assessment

The variability in terminology and parameters measured in association with a treadmill based walking test extends to the method of assessment. Constant load treadmill testing (one that does not increase in speed or gradient), has been criticised due to the potential inability of some patients to reach claudication symptoms while exercising at a low workload (134). Such an issue was highlighted by Gibellini et al (119) who, using a constant load treadmill test of 3km/hr with 0° gradient, noted that 70% of patients were asymptomatic (defined as walking PFWD >1000m) after 6 months of supervised exercise. In support of these results, although not reporting any patients to be asymptomatic, after a similar exercise program, Spronk et al (135) used a constant load treadmill test of 3.5km/hr with 0°gradient and achieved a mean PFWD of 899m. It is likely that the additional workload induced by a 0.5km/hr increase in

treadmill speed can account for the slightly shorter distances achieved by Spronk et al (135) compared to Gibellini et al (119). Further increments in workload were employed by both Kakkos et al (136) and Mika et al (127) who used gradients of 10° and 12° respectively. Not unexpectedly given the markedly increased workload induced by such a gradient, they reported much shorter PFWD, mean of 70m after 6 months exercise in Kakkos et al (136) and 191m after 3 months in Mika et al (127). While these studies help to overcome some of the criticism aimed at constant-load treadmill testing, further criticism relating to reproducibility has also been published. In 1969, Alpert et al (137) reported a coefficient of variance (CV) of 8% for constant load treadmill testing, while more recently values in the range of 30-40% have been published (138). Several variations existed between these trials which may account for the observed differences. Alpert et al (137) had the treadmill set at 4.6km/hr at a gradient of either 8% or 16%, while, participants were allowed a preliminary "familiarisation" test to learn how to walk in a relaxed and efficient manner on the treadmill. In Gardner et al (138), the treadmill speed was slower (3.2km/hr) and no attempt at familiarisation was described. The concept of familiarisation is an important one considering that the biomechanics required to effectively use a treadmill are quite different to normal land-based walking (122). Supporting this is the fact that initial walking assessment has previously been shown to provide significantly lower values than subsequent tests (137). Of the 18 studies to assess the impact of treadmill based walking on walking performance compared with controls, only five have reported using familiarisation techniques (49, 124-126), thus providing the potential that reported baseline values of walking assessment are substantially under-estimated, with implications on the accuracy of statistical analyses.

Although only 1/18 (127) of included trials used constant-load testing, certain aspects of the graded-load treadmill assessment protocols, employed by the other 17 studies, also require consideration. Such protocols are characterised by initially low work demand with a

subsequent increase in speed and/or gradient at set time intervals. Like constant load testing, there is marked heterogeneity between these protocols, which ultimately influence the ability to compare and contrast results within trials. The graded test has been suggested to accommodate patients with IC with various disease severity (139). Furthermore, Gardner (138) proposed that intra-patient variation in walking performance should be reduced due to a gradual increase in metabolic demand and the ability of all patients to reach a maximal walking performance that is more closely associated with their disease severity. He supported this statement with data from his own study in 1991 (134) suggesting a CV of only 13%, even without employing a familiarisation technique. He proceeded to conclude that only one graded test is required to obtain reliable measurements of walking performance, while three tests are necessary when utilising constant-load tests. Despite this, highlighting a role for familiarisation to the treadmill, Sanderson et al (116) reported a CV of only 6% after providing patients with exposure to the treadmill before testing commenced. Such a value is comparable with that of Alpert et al (137), who as mentioned previously, used familiarisation in patients undergoing constant-load testing and reported a CV of 8%.

Also in conflict to the conclusion of Gardner are results from Cachovan et al (140), who after familiarisation, demonstrated that constant load and graded treadmill tests were equally reproducible, albeit with a CV of over 20%. Although such variation seems large, a spontaneous intra-individual fluctuation of up to 25% is regarded as a reasonable limit for a stable walking distance (140). A fact not lost on Murphy et al (49) who excluded patients with between test variation of >25%. This concept highlights the inherent flaws associated with the assessment of walking performance. On any given day, there may be fluctuations in the mental and physical condition of the patient and perhaps most importantly, the subjective nature of claudication pain must be considered as an uncontrollable variable.

Despite the glaring need for standardisation of techniques and the obvious problems associated with reproducibility, treadmill testing remains the preference of choice for the assessment of walking performance after exercise intervention. This is demonstrated by a recent Cochrane review protocol (141) dedicated to determine the effects of all types of exercise on IC. Trials without treadmill assessment of walking performance were excluded. Concern over this method however, was first expressed by Watson et al in 1997 (131), who suggested that treadmill testing was somewhat artificial and did not represent the day to day walking ability of a patient. More recently, McDermott et al (122) further questioned the validity of treadmill testing citing its susceptibility to a learning effect as a major negative of such a technique.

To put this into perspective, studies assessing the biomechanics of land-based versus treadmill walking have reported differences in muscle activity, joint movements and walking rate between the two modalities (142). The impact of these differences is highlighted by Watson et al (131) who showed that at baseline assessment, most patients were able to walk substantially further at their own speed in the corridor than on the treadmill at a slower speed. This supports the previously mentioned finding of Alpert et al (137) in 1969 that initial walking assessment often provides significantly lower values than subsequent tests. It is likely that the increased work load induced by unfamiliar biomechanic techniques leads to earlier onset of claudication symptoms, thus explaining these findings. As has been discussed, this can, in part, be overcome after one or two sessions of orientation to treadmill technique. It therefore seems reasonable to suggest that adaptation to treadmill based walking should continue among patients who are involved in a prolonged treadmill based exercise intervention, thus bringing into question whether or not the observed improvements in walking assessment in the majority of studies are in fact due to physiological adaptations or indeed simply the ability to adapt to the method of treadmill walking.

The six minute walk test (6MWT) is a well validated, reproducible technique that is an objective measure of functional exercise capacity (143). Importantly, it is a land based test, making it an accurate representation of actual functional ability and excluding the potential for a learning effect to confound results (143). It has been used as the method for primary outcome measurement in McDermott et al (122), while Gardner et al (114) and Tsai et al (115) have used it as a secondary outcome to supplement results from the treadmill based primary outcome assessment. McDermott et al (122) showed small but significant improvements in the total distance walked in the 6MWT after a 6 month treadmill based exercise intervention (327m to 348m), as did Tsai et al (216m to 261m) (115). While it could be speculated that such changes are relatively small compared with the improvements in walking time (both pain free and maximal) from treadmill based assessment, the different units used and time limitation of the 6MWT prevents direct comparison and meaningful conclusions from being made. Only Gardner et al (114) used the same unit of measurement for both methods, with interesting results. While both treadmill and 6MWT produced significantly improved values for PFWD compared with the control group, the treadmill based test resulted in an improvement from 172m to 402m, compared with that of the 6MWT from 175m to only 252m. Although the two sets of values have not been compared statistically, there certainly appears to be a much greater improvement in the results derived from the treadmill test. This may be reflective of the learning effect associated with treadmill walking and brings into question the accuracy of treadmill based assessment, particularly in the context that the primary goal of an exercise intervention is to enable patients to walk further overground rather than on a treadmill.

A slightly different perspective is provided on this through careful analysis of an article from Treat-Jacobsen et al (121). It was mentioned earlier in this discussion (*See section 1.3.2.2.4* Exercise intervention) that 75% of participants in the control group in this study reported

participating in outside exercise (defined as moderate intensity exercise, usually walking, for a minimum of 30 minutes) at least three times per week. Despite these patients undertaking a similar volume of exercise to those who are undertaking a formal supervised exercise intervention, treadmill assessment of walking performance failed to demonstrate an improvement from baseline in these patients. Although strong evidence is provided by Fokkenrood et al (144) who, in a 2013 Cochrane review, suggested that supervised exercise therapy is clinically superior to non-supervised regimens, all trials included in this analysis used a treadmill test as one of the primary outcome measures. It is therefore possible that patients undertaking non-supervised exercise are not experiencing long periods of exposure to the treadmill and as a result, not experiencing the learning effect.

From the above discussion, it seems that the 6MWT may be a more appropriate measure of the actual physiological improvement to walking performance induced by exercise. If this is proven to be the case, much of the current literature would become meaningless and the value of exercise programs for patients with IC would need revisiting. Furthermore, if 6MWT is found to be a more appropriate measure then results would suggest that there may not be as much physiological improvement in walking performance as previously proposed. Of course, before reaching any strong conclusions about the merits of each method of assessment, additional and more focussed work needs to be undertaken to compare the two.

1.3.2.2.8 Statistical analysis

Perhaps the biggest statistical limitation to trials of exercise rehabilitation is the challenge of recruitment and compliance. Such a challenge has previously been identified with respect to cardiac rehabilitation, with the suggestion that lower rates of utilisation of rehabilitation opportunities are found among women, older individuals and those with lower socio-economic status (145). In the trials of patients with PAD, the major issues ultimately leading to exclusion were time and transport constraints and other cardio-respiratory co-morbidities

that meant treadmill-based exercise was contra-indicated. Furthermore, the inclusion criteria for each study were quite strict, particularly with respect to the baseline pre and post exercise ABPI values required and the need to replicate pain free walking performance within a CV of <10% (49) to <25% (122) on a screening treadmill test on consecutive days in some studies. Highlighting the extent to which recruitment was a challenge is McDermott et al (122) who assessed 1009 patients and only randomised 156 patients (15%) across three study arms and Murphy et al (49) who screened 999 patients and randomised 119 (12%) across four study arms. Randomisation techniques were generally acceptable and computer-based, although 8/18 studies were unclear (112-115, 123-125, 127).

The numbers in each trial were further compromised by issues with compliance which needs to be considered as both the number of exercise sessions attended and number of withdrawals from the program as a whole. With respect to the number of sessions attended, 7/18 studies reported values (113-115, 120-122, 125). Gardner et al (113) best highlights the intra-study variation of this figure, reporting a range of 54-100% of sessions attended. In each case, such variation was not adjusted for in statistical analysis, and numbers were too small to calculate a dose-response to exercise volume.

Reasons for withdrawal from the program as a whole were similar to reasons limiting recruitment (ie time, transport and health), however, the number of withdrawals was quite small. This is likely to reflect the perceived health benefits of exercise which participants experienced soon after commencement of such a program. Only Gelin et al (128), reported a high dropout rate, with 59% of patients completing the walking program. This may, in part, account for the lack of demonstrated improvement in walking performance derived from this study.

Generally speaking, intention to treat (ITT) analysis was undertaken, albeit of a modified nature. Participants who withdrew from the program usually failed to return for post-intervention assessment and were therefore excluded from analysis. The purpose of ITT analysis was therefore mostly limited to the consideration of participants who were non-compliant with the SET but did not withdraw from the program as a whole. Only Hiatt et al (112) excluded non-compliant participants from the final analysis, effectively undertaking a per-protocol analysis only.

Arising from difficulties with recruitment and compliance is the issue of small sample size. Gelin et al (128) randomised the largest number of participants including 75 in the treadmill based exercise group and an equivalent number of controls. At the other end of the spectrum, Wood et al recruited only 13 participants in total for randomisation between both an intervention and control group. As an overview, 10/18 groups (112, 113, 116-118, 121, 123-126) recruited less than 20 patients to each arm of their study. These studies included 4/5 (123-126) which failed to demonstrate significance between treadmill and control groups, raising the possibility of a type 2 statistical error in these studies, masking the potential benefits of exercise.

1.3.2.3 – Summary of exercise interventions

To summarise, there is certainly evidence to support a role for a treadmill based walking intervention in the treatment of patients with IC, however, it may not be as compelling as what was previously thought and the challenge lies in identifying those who will and will not respond positively to such an intervention. The small number of patients included and marked heterogeneity of relevant studies makes it difficult to make meaningful comparisons between studies, limiting the potential to reach significant conclusions. To improve such potential, it should be recommended that universal guidelines be introduced pertaining to the implementation of exercise based interventions, particularly with respect to method and

reporting of outcome assessment. Furthermore, additional work is required to compare the value of treadmill based assessment of walking ability which maybe confounded by the learning effect or the land-based 6MWT which may in fact prove to be a more accurate measure of functional walking performance.

1.3.3 Mechanism of response to exercise

1.3.3.1 The endothelium

The clinical response to exercise in patients with IC maybe due to an array of systemic and local biological adaptations that occur during the period of exercise, or it may be a result of "learned" technique and improved pain tolerance. Given the impact and benefit of exercise on endothelial function across a broad range of patient demographics and the established presence of endothelial dysfunction in patients with PAD (146), it seems reasonable to speculate that alteration of the endothelial phenotype may be a critical central mechanism through which the body regulates its response to exercise in patients with IC. In fact, it has been proposed that such a phenotype not only precipitates the onset of atherosclerosis and PAD but subsequently contributes to the clinical status and disease severity (147).

The key to this statement is an understanding of the endothelial regulation of vascular homeostasis (*See section 1.2* Role of the Vascular Endothelium – Cellular Mechanisms*).* To reinforce the previous discussion, in the state of ED, NO bioavailability is reduced and vascular homeostatic mechanisms are impaired (54). Most significantly, in patients with PAD, the normal vasodilatory vasomotor response to exercise induced shear stress is impaired, often resulting in vasoconstriction and subsequently worsening the anatomical degree of stenosis associated with atherosclerotic plaque, subsequently impacting on walking performance (147). Furthermore, vascular remodelling and compensatory collateral formation (angiogenesis) is dependent on NO bioavailability and has been shown to be blunted in

patients with ED (148), while the prothrombotic and pro-inflammatory state associated with ED may result in thrombosis and plaque rupture with subsequent lesion progression and worsening of symptomatology (147).

In short, it is speculated that the sheer stress associated with regular aerobic exercise may lead to an adaptive response to alter the intrinsic responsiveness of eNOS, subsequently improving NO bioavailability and endothelial function (149). Further benefit in endothelial function is likely to be derived from the exercise induced improvement of cardiovascular risk factors, which are linked with a pro-atherogenic endothelial phenotype (105).

1.3.3.1.1 Angiogenesis and VEGF

Numerous studies have demonstrated no improvement in ABPI's from baseline to the completion of SET (112, 114, 124, 125). In addition, Gardner et al (113, 114) demonstrated no significant improvement in resting calf blood flow after an exercise program. Combined, these findings suggest a lack of collateral formation with a baseline level of anatomical stenosis, not influenced by changes in vasomotor tone while in the resting state.

Such a lack of improvement in the area of vascular remodelling is perhaps not surprising considering the multi-factorial nature of this process which requires a balance between angiogenic versus angiostatic factors, many of which are released in response to hypoxia, the most important initiator of angiogenesis. Interestingly, levels of vascular endothelial growth factor (VEGF), a key factor involved in the initiation of angiogenesis (150), have been shown to increase following an acute bout of exercise to pain in patients with claudication (151). This seems logical given that such exercise is inducing hypoxia in these patients, stimulating the response of factors such as VEGF, although Hudlicka et al (152) also points out the importance of shear stress and NO bioavailability, independent of hypoxia, in promoting angiogenesis through the increased expression of VEGF. It is possible therefore that SET in

these patients may lead to microvascular angiogenesis of capillaries to improve tissue perfusion and symptoms without manifesting as an improvement in ABPI or resting calf muscle blood flow. This clinically undetectable improvement in angiogenesis has been demonstrated as part of the adaptive response to exercise training in the rat model (153). Furthermore, it should be mentioned that major arteriogenesis is a chronic process and therefore unlikely to occur within the time frame specified for the exercise intervention in these trials. It may be detected after prolonged (>1 year) exercise programs, however, this remains to be seen.

In contrast to the above studies is more recent work from Jones et al (154). Although demonstrating an increase in VEGF expression and levels, Jones et al have proposed that an anti-angiogenic sub-type of VEGF exists and can account for such an increase, while levels of the pro-angiogenic VEGF are reduced following exercise training, potentially preventing the onset of angiogenesis. Further investigation is therefore required to investigate this concept and to understand whether or not exercise in patients with PAD is pro or anti-angiogenic.

1.3.3.1.2 Platelet function

Coagulation and inflammation are other areas of vascular homeostasis which are regulated by the endothelium (51). A pro-thrombotic, pro-inflammatory state is a key feature of ED (54) which may contribute to the symptomatology of PAD and may also be modifiable through an improvement in endothelial function induced by an exercise program, thus contributing to the mechanism through which exercise exerts its beneficial effect on such patients. The impact of improved endothelial function on systemic inflammatory burden is difficult to quantify given the confounding effect of the pro-inflammatory ischaemia-reperfusion injury which occurs concurrently with the onset of claudication symptoms (*See section 1.3.4.2* Exposure to Ischaemia-Reperfusion Injury).

Coagulation, or more specifically, platelet function, is also somewhat difficult to assess given the challenges associated with the study of platelet aggregation and activation (155). It is recognised that patients with PAD have increased platelet activity (156), which is expected given the link with endothelial dysfunction and associated pro-thrombotic state. Furthermore, in patients with CAD, physical activity has been shown to increase platelet activity and aggregation, potentially worsening the risk of myocardial infarction (157). Arosio et al (158) investigated the impact of SET on platelet function in participants with IC. After 2 weeks of physical training, platelet activity was reduced ex vivo. Although this was not the case with testing performed in vivo, possibly due to the short duration of the intervention, such a result exhibits similarities to findings in young adults, who, after a moderate intensity physical training program, were found to display reduced platelet aggregation and adhesion at rest and in response to exercise (159). These findings are presumably due to improvements in endothelial function and NO bioavailability associated with exercise (160). Significantly, high intensity exercise has been shown to induce a detrimental increase in platelet activity in healthy individuals (159). Although such a response was attenuated after training, this highlights the importance of the prescription of exercise programs of appropriate intensity in patients with PAD.

1.3.3.2 Adaptations in skeletal muscle

While exercise in patients with PAD may improve the health of the endothelium and therefore result in improved vascular homeostasis and symptomatology, specific adaptations in the affected skeletal muscle have also been proposed as contributing factors by which exercise can improve walking performance in patients with IC (161).

1.3.3.2.1 Metabolism

Claudication pain is essentially due to an imbalance in the supply and demand for oxygen, leading to impairments in muscle metabolism and oxygen extraction and ultimately anaerobic

glycolysis and lactic acid accumulation (161). To help overcome this problem, exercise programs in patients with IC have been shown to induce skeletal muscle enzymatic adaptations, leading to up-regulation of oxidative enzymes and improvements in muscle oxidative metabolism (162). Specifically, carnitine is a quaternary ammonium compound that plays a key role in glucose oxidation and energy production through the Krebs cycle (163). In patients with IC, transient ischaemia induced by exercise leads to a state of metabolic stress and alteration of carnitine metabolism with subsequent accumulation of its intermediates, including acylcarnitine (44, 46). A reduction of acylcarnitine levels, reflective of improved carnitine metabolism and skeletal muscle oxidative capacity, has been demonstrated to correlate with improvements in walking performance following 12 weeks of treadmill based exercise training (164).

1.3.3.2.2. Muscle recruitment

Preliminary work has also suggested that skeletal muscle recruitment may play a role in the response of patients with IC to exercise training (165). Such work was based on the concept that in healthy individuals, inhibitory signals arise from exercising skeletal muscle and feedback to the central nervous system to signal fatigue and reduce skeletal muscle recruitment (165). In patients with PAD, angioplasty with subsequent improvement in flow has been shown to increase muscle activity of the affected leg (166), while three weeks of SET which improved walking performance was found to significantly increase lower limb muscle activity (median frequency measured by electromyography from 70 to 78Hz, $P=0.045$), although still not to normal levels, as compared with healthy, age-matched controls (167). In the context of other results presented in this study, the authors concluded that central nervous system drive increases following SET and factors other than ischaemia and physical inactivity underlie the abnormal skeletal muscle activity in patients with PAD (167).

1.3.3.2.3 Calpain system

Exercise is associated with a systemic state of protein catabolism and exercise induced increases in contractile activity results in significant changes in muscle structure and function (168, 169). While the exact mechanism for such changes is unclear, alterations in skeletal muscle calcium homeostasis have been proposed to activate calcium dependent non-lysosomal cysteine proteases such as those of the calpain system (168-170).

The calpain system consists of 14 different members and an endogenous calpain inhibitor known as calpaststin (171). Skeletal muscle expresses 2 ubiquitous calapins; u-calpain (activated by micromolar concentrations of calcium) and m-calpain (activated by millimolar concentrations of calcium), as well as muscle-specific calpain-3 (172, 173). Although their exact physiological roles remain poorly understood, they have been implicated in both muscle apoptosis and myogenesis as well as sarcomeric remodelling (173). Underlining the importance of the calpain system in muscle structure and function is the fact that a mutation in the gene coding for calpain-3 results in limb-girdle muscular dystrophy type 2a (170, 171, 173).

At resting intra-cellular calcium concentrations, the calpains are present in their inactive form. Following a stimulus such as exercise, the intra-cellular calcium concentration increases, leading to calcium binding, a conformational change and subsequent autolysis to activate the calpain molecule (168, 169). Once active, calpains have been shown to cause degradative changes in skeletal muscle myofibrils and subsequent morphological damage (168, 169, 171). This is likely to be due to ubiquitous calpain (u- and m-calpain) activity on the basis that calpain-3 has been proposed to be a signalling protease rather than actively involved in the turnover of myofibrillar protein (171). The statement is supported by muscle wasting in patients with type 2a limb girdle muscular dystrophy, despite loss of calpain 3 activity as induced by the genetic mutation mentioned previously (171). Although m-calpain

in adult skeletal muscle seemingly requires supra-physiological calcium concentration for activation, it is possible that its activity may be influenced by factors other than calcium, including certain phospholipids, which may lower the calcium concentration required for activation (174). Supporting this is the increased skeletal muscle u- and m-calpain activity that was demonstrated in healthy rats, immediately after running to exhaustion (169). More recent findings are in conflict to this with Murphy et al (173) subjecting human volunteers to exhaustive cycling exercise before harvesting vastus lateralis muscle which did not result in increased levels of u-calpain activity. Possible explanations for the discrepancy between trials is the human versus rat model, the fact that volunteers in Murphy et al (173) were already endurance trained and therefore adaptation of the calpain system may have already occurred and the conclusion from Murphy et al (173) that the exercise protocols used may have resulted in a type of muscle fatigue associated with accumulation of intracellular metabolites with an inhibitory effect on calcium release, thus, not driving calpain activation.

Further support for the role of the calpain system in muscle turnover is provided by work in a rat model, in which an increase in calpain activity associated with isoproterenol-induced cardiac hypertrophy resulted in extensive myofibrillar damage which was attenuated in rats who were pre-treated with a cysteine protease inhibitor E64 (175). This brings to light the significance of the endogenous calpain inhibitor, calpastatin, an important regulator of the calpain system. The complexity of such regulation is highlighted by the fact that calcium levels also regulate binding of calpastatin to calpain, while activated calpain is capable of degrading calpastatin (171). In a rare study to assess the role of calpain and calpastatin together, the catabolic, muscle wasting state associated with sepsis in a rat model was linked with increased calpain activity, proposed to be due to reduced calpastatin activity (174).

The role of calpain in muscle structure and function seems not to be limited just to the catabolic state of wasting. An influx in neutrophil numbers has been reported in skeletal

muscle following endurance exercise, likely representing the early inflammatory and regenerative response to exercise induced muscle injury (176). The calpain system has been reported to be chemotactic to neutrophils, in part initiating such a response (177). Furthermore, elevated levels of u- and m-calpain expression have been implicated with the fusion and differentiation of primitive muscle cells (168). Such an observation is strengthened by the knowledge that calcium levels play an important role in the regulation of cellular mitosis and proliferation (178). It is possible therefore that the calpain system may be a key component of both muscle injury and in response to exercise and the subsequent muscle repair process. Its dual role means it may be critical in explaining the adaptive response associated with prolonged exercise training.

In order to facilitate a better understanding of the calpain system and its activation, the physiological role of calcium in skeletal muscle and the impact of exercise on calcium concentration must be considered. In the quiescent state, intracellular calcium concentration in skeletal muscle is carefully maintained, largely within the sarcoplasmic reticulum (an organelle contained in striated muscle cells) (179). When the muscle cell is stimulated by a central nervous system message transmitted as an action potential to the neuromuscular junction and subsequently the cell itself, calcium is released by the sarcoplasmic reticulum through voltage gated calcium channels. Such an influx of calcium precipitates skeletal muscle contraction through the process known as excitation-contraction coupling (179). These transient increases in calcium concentration are not sufficient to activate calcium dependent proteases, however, it has been suggested that exercise results in further and more prolonged elevation of calcium concentration by calcium influx through either stretch activated channels or high tension ruptures of the sarcolemma (muscle cell membrane), with resultant activation of calcium dependent proteases such as the calpain system (168).

From a pathological perspective, acute ischaemia has also been demonstrated to increase intracellular calcium concentration (180). This is relevant to patients with PAD and particularly those with IC, in whom transient and repeated ischaemic episodes induced by exercising to the onset of pain, may result in markedly elevated calcium concentrations through the cumulative impact of both exercise and ischaemia, with subsequent high levels of calpain activity. In fact, hypoxia has been shown to induce calpain activity in retinal endothelial cells (181). In patients with IC, this could potentially result in muscle wasting, a concept that is supported by work from Tsai et al (182), who, using a rat model of chronic ischaemia demonstrated ischaemia-induced muscle atrophy to be more severe in exercise-trained rats than untrained rats, suggesting that for individuals with impaired vascular conditions, exercise training might not be beneficial in maintaining muscle mass. Studies of human subjects with PAD have revealed that lower extremity ischaemia has a detrimental effect on calf muscle skeletal area when compared with age-matched patients with normal lower limb perfusion status (183), however, the impact of exercise on muscle mass in these patients is yet to be investigated.

To complete the discussion on the calpain system, high levels of calpain activation have been implicated with a failure of VEGF induced angiogenesis (184), suggesting that a regulatory pathway exists between the calpain system and angiogenic pathways. This may help to further explain the absence of clinically detectable vascular remodelling, discussed previously (*See section 1.3.3.1.1* Angiogenesis and VEGF), in patients with IC after the completion of exercise interventions.

In summary, it seems therefore that the ability of the calpain system to adapt to variations in intracellular calcium levels, may be a key factor in preventing muscle wasting as well as determining and explaining response to exercise programs in patients with IC.

1.3.3.3 Psychological role of exercise

Consideration must also be given to the possibility of a placebo or psychological response to exercise which may, in part, explain the improvement in walking performance associated with an exercise program. The concept of pain is complex and very subjective defined by the international association on the study of pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (185). It can be influenced by a host of factors including physiological, psychological and environmental, a fact that is highlighted by the well-established differences in pain perception and tolerance threshold associated with ethnicity, genetics and sex (186). Pain can be modified by medication and psychological support to the extent that in a study of chronic pain sufferers, pain was shown to be greatly reduced or resolved during participation in certain activities (187). Furthermore, highlighting the role of placebo is a study in which 35% of patients reported a significant analgesic effect of a saline injection that was believed to be morphine (187). Relevant to patients with IC, it has been shown that the patients who demonstrated the greatest improvement in walking performance were those of the belief that exercise would lead to an improvement in walking status (188). It is also possible that overcoming avoidance of painful stimuli, through repeated exposure to claudication pain, as is the case with a treadmill based exercise program, may lead to an improvement in pain threshold and the ability to tolerate pain. In support of this, Gardner et al (189) identified that the ability of patients to walk further despite pain was an independent predictor of greater improvement in PFWD following an exercise intervention. Of course, this may be as much due to a physiological adaptation to pain as it is psychological. Parr et al (165) have suggested that the plasticity of the motor cortex is such that a reduction of motor fiber recruitment may occur in response to pain, enabling improved tolerance and better functional results.

1.3.4 Detrimental effects of exercise in IC

The above discussion demonstrates work into the elucidation of the mechanism by which supervised exercise exerts beneficial effects on patients with IC. Little consideration however, has been given to the potential detrimental effects.

1.3.4.1 Pro versus anti-inflammatory response

A pro-inflammatory response to acute exercise in healthy individuals is well documented (190). Active stretching of skeletal muscles during exercise increases the expression of pro-inflammatory cytokines which in turn leads to endothelial activation, expression of CAM's and recruitment of neutrophils (190). This is a transient physiological response in isolated episodes of exercise providing a means of repair to skeletal muscle damage sustained during exercise (190). Sustained, repeated bouts of exercise however, lead to an adaptation of this pro-inflammatory response, ultimately resulting in an increased expression of anti-inflammatory molecules which are associated with numerous beneficial physiological effects including enhancement of the functional capacity of the immune system (190). Such an adaptive response would also contribute to the exercise induced augmentation of endothelial function and associated beneficial effects that have been previously discussed *(See section 1.3.1* Benefits of exercise*).*

This adaptive anti-inflammatory response to exercise has led to the adoption of exercise as a key treatment for several systemic inflammatory conditions such as metabolic syndrome, chronic heart failure and CAD (97). To highlight the effectiveness of this, Goldhammer et al (191) subjected patients with CAD to a 12 week exercise program consisting of moderate to high intensity upper and lower limb aerobic exercises. The result was a reduction in pro-inflammatory cytokines including C-Reactive Protein (CRP), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) and an increase in the anti-inflammatory cytokine Interleukin-10 (IL-10). Given the contribution of inflammatory cytokines to the onset and progression of

atherosclerosis (192), such a finding is significant for patients with vascular disease, most of whom have an underlying systemic inflammatory burden already associated with their disease process (192, 193).

On the strength of this argument, it seems reasonable that patients with PAD would also experience a beneficial anti-inflammatory response to exercise, however, such an assumption does not take into account the role of ischaemia-reperfusion injury (IRI).

1.3.4.2 Exposure to Ischaemia-Reperfusion Injury

Ischaemia-reperfusion injury is defined as "cellular damage after reperfusion of previously viable ischaemic tissue" (194). It is characterised by an inflammatory cascade and increased microvascular permeability that in severe cases can lead to multi-organ dysfunction syndrome and death (194). At a biochemical level, ischaemia leads to an inability of the cell to resynthesise energy rich phosphastes including adenosine triphosphate and phosphocreatine, leading to the accumulation of hypoxanthine (194). Under normal physiological conditions, hypoxanthine is metabolised to xanthine via xanthine dehydrogenase. During ischaemia however, xanthine dehydrogenase is metabolised to xanthine oxidase which uses oxygen as its substrate. In ischaemic conditions, it is therefore unable to catalyse the conversion of hypoxanthine to xanthine (194). During reperfusion and the re-introduction of oxygen to the cell, xanthine oxidase converts the excess hypoxanthine to toxic ROS such as superoxide anions and hydrogen peroxide (194). These are capable of causing tissue injury and inflammation through several mechanisms including lipid peroxidation of cell membranes and activation of leukocytes and other transcription factors (194).

Intermittent claudication is essentially repeated low grade IRI. At rest lower limb skeletal muscle perfusion is adequate to meet metabolic demands, however, exercise increases such

demands which ultimately cannot be met due to limitations in blood flow associated with the disease process. This leads to claudication pain being manifest from ischaemic muscles. During recovery from exercise, the metabolic requirements of the muscle decrease and pain resolves as adequate blood flow is once again restored to the ischaemic muscle ie reperfusion.

Interestingly, repeated, transient exposure of tissues to ischaemia has been proposed to play a protective role against prolonged ischaemia-reperfusion injury. This concept, known as ischaemic preconditioning, is thought to increase extracellular adenosine levels, a molecule that confers a protective effect to tissue during ischaemia (195). Experimental models of ischaemic preconditioning have demonstrated a reduction in myocardial inflammatory markers, while clinical attempts at such preconditioning have been associated with a reduction in hepatocyte damage following partial hepatectomy (194).

This raises the possibility that repeated low-grade IRI experienced by patients with IC during SET is in fact stimulating an adaptive anti-inflammatory response allowing a higher threshold for tolerance of IRI that is reflected by improvements in PFWD. In contrast, it would seem equally feasible that repetitive exposure to low-grade IRI may dampen or even override the protective anti-inflammatory effect of exercise on patients with IC, placing these patients at an increased risk for disease progression and future cardiovascular events.

1.3.4.2.1 Contribution of IRI to systemic inflammatory burden

Several studies have been undertaken to highlight the acute impact of IRI on the inflammatory biochemistry in patients with IC. Edwards et al (196) initially added support to the concept that atherosclerotic disease is associated with a pro-inflammatory state, by demonstrating that baseline neutrophil count was significantly higher in patients with IC than healthy controls ($5.6x10^6$ versus $2.8x10^6$ cells, $P<0.05$) Furthermore, after 5 minutes of treadmill based exercise, neutrophils in patients with IC had risen to significantly higher

levels when compared with baseline ($5.6x10^6$ to $7.1x10^6$ cells, $P<0.05$). These had returned to baseline levels after resting for 15 minutes. Neutrophil levels in controls were unchanged, implicating neutrophils in the pathogenesis of IRI. It is likely that through a complex mechanism involving ischaemia induced leukocyte-endothelial cell adhesion and endothelial transmigration, neutrophil chemotaxis occurs (196). Neutrophil activation then occurs through a poorly understood mechanism, followed by degranulation via cellular activation of protein kinase C. Activated neutrophils are larger and stiffer than the inactive form, leading to an obstructive phenomenon in capillaries, with subsequent prolonged sub-clinical ischaemia even during the presumed reperfusion phase (196). Degranulation of neutrophils precipitates release of various pro-inflammatory products of the arachidonic acid pathway and proteolytic enzymes (197). One such enzyme is neutrophil elastase (NE), a serine protease with a broad substrate specificity but significantly, at a local endothelial level, the ability to destroy the endothelial cell basement membrane, leading to increased microvascular permeability (197). Relevant to PAD, in a large scale population based study, NE levels are increased at baseline relative to healthy controls (40.8ng/ml versus 32.8ng/ml, $P=0.002$) (198). Further to this, Turton et al (199) have demonstrated a significant rise in NE (44.6ug/L to 86.7ug/L, $P<0.05$) in association with a brief but significant neutrophilia in patients with IC undertaking a single bout of exhaustive treadmill-based exercise.

Confirming the importance of neutrophils and their products is epidemiological evidence implicating neutrophil count as a risk factor for future cardiovascular events (200). Additionally, Spark et al (201), in a cohort of vascular patients with CCLI, identified that a high neutrophil-lymphocyte ratio was predictive of shorter survival, compared to those with low to normal values.

Preceding the activation of neutrophils and stimulating the expression of adhesion molecules responsible for leukocyte-endothelial cell adhesion, are several pro-inflammatory cytokines

including IL-1, IL-6 and TNF-α (197). These are typically released by macrophages and monocytes at sites of tissue injury and form part of a large family of cell-signaling, immuno-modulatory molecules (190). Several studies have assessed levels of these cytokines both at rest and acutely following a bout of exercise in patients with IC (151, 202, 203). Palmer-Kazen et al (151) measured IL-6 and TNF-α from plasma samples of patients with IC and healthy controls taken at various time-points up to two hours following completion of treadmill walking to MWD. While there was no difference in cytokine values between the groups at baseline, levels of IL-6 continued to increase significantly from baseline two hours after exercise in both patients with IC and healthy controls (exact values for mean and standard deviation not provided). TNF-α levels did not significantly change from baseline in either group. Work from Andreozzi et al (202) provided some further insight into the time-related increase in IL-6 levels, albeit somewhat in conflict with the results of Palmer-Kazen et al (151). Andreozzi et al (202) showed that differentiation between patients with moderate and severe IC (Rutherfords 2 and 3 respectively) led to contrasting trends. Although a maximal treadmill test led to significantly increased IL-6 levels from baseline to cessation of exercise in all patients with IC (5.97pg/ml to 8.38pg/ml in patients with moderate IC and 6.98pg/ml to 9.99pg/ml in patients with severe IC) and healthy controls (4.84pg/ml to 5.28pg/ml), after 15 minutes of recovery, IL-6 levels in patients with moderate IC (8.38pg/ml to 7.25pg/ml) and healthy controls (5.28pg/ml to 4.91pg/ml) had subsided significantly, while in patients with severe IC (9.99pg/ml to 11.94pg/ml), levels continued to rise significantly. Similar trends were observed with respect to IL-1. Two reasons may explain the differences observed between Palmer-Kazen et al (151) and Andreozzi et al (202). The cohort of patients in Palmer-Kazen et al is not well defined with respect to severity of claudication and may contain predominately patients with severe IC. Furthermore, participants in Palmer-Kazen et al (151)continued exercise to MWD, thus greatly increasing the duration of ischaemia and

potential for subsequent inflammatory response to IRI, compared with those in Andreozzi et al (202) in who exercise was terminated at the onset of claudication pain, limiting exposure to IRI. Signorelli et al used a similar method to Andreozzi et al (202) and reported levels of IL-6 and TNF-α. They found levels of both IL-6 and TNF-α were significantly higher immediately following exercise in both patients with IC (IL-6: 11.81ng/ml to 15.8ng/ml and TNF-α: 14.48ng/ml to 26.65ng/ml) and controls (IL-6: 7.30ng/ml to 8.15ng/ml and TNF-α: 9.32ng/ml to 11.5ng/ml. This is consistent with the mechanism of cytokine release arising from exercise in healthy individuals (190), however, the magnitude of observed increase was significantly higher in patients with IC as compared to controls, suggesting that even cessation of exercise at the onset of pain is sufficient to induce at least some degree of IRI and increased inflammatory response. Collins et al (204) published data to suggest that IL-6 did not acutely increase in response to exercise. Interestingly, in this study 42% of patients with IC returned values below the detectable range of the enzyme-linked immunosorbent assay (ELISA) kit used. Similar results were published by Fiotti et al (192). In the 1999 manuscript in *Atherosclerosis*, Fiotti et al (192) reported that immediately following an acute bout of exercise, IL-6 levels in both patients with IC and controls were undetectable. Furthermore, IL-1 and TNF-α remained at baseline levels. The authors have not proposed a mechanism for such a finding and these results should certainly be viewed with caution, particularly given the small numbers in each group (n=8) and the very low-intensity (0% slope at 3km/hr for 30 minutes with a 1 minute rest every 5 minutes) exercise regimen that was used in which it is unclear whether or not claudication pain was even induced. Of interest and unique to the study of Fiotti et al (192) is the finding that levels of the IL-1 receptor antagonist, but not the receptor itself, were elevated in patients with IC and controls immediately following exercise. It was proposed that such upregulation may be part of a longer term adaptive anti-inflammatory response, a concept supported by further work in healthy individuals

suggesting that IL-6 stimulates the upregulation of IL-1 receptor antagonist and low muscle glycogen stores exert a positive feedback effect (205).

Undoubtedly, further large-scale research is warranted to determine the exact nature of the inflammatory response to acute exercise and IRI in patients with IC. While the current body of evidence remains in favour of an acute pro-inflammatory response to exercise contributed to by both the normal physiological response to exercise and made worse by the exposure to IRI, the duration of the acute inflammatory response to exercise beyond two hours remains unclear.

1.3.4.2.2 Impact of acute exercise on endothelial function in IC

An earlier discussion on endothelial function highlighted its role in contributing to the chronic pro-inflammatory state associated with atherosclerotic disease (*See section 1.2.4.4* Endothelial Dysfunction and Atherosclerosis*).* The increased endothelial expression of CAM's is associated with a dysfunctional endothelium and is known to precipitate atherosclerotic plaque formation and a systemic pro-inflammatory response (206). Several studies have also investigated the impact of exercise in patients with IC on levels of CAM's (158, 203, 207).

As well as the assessment of IL-6 and TNF-α (*See section 1.3.4.2.1* Contribution of IRI to systemic inflammatory burden*),* Signorelli et al (203) also measured levels of VCAM and ICAM at rest and after exercise. At rest both VCAM and ICAM were higher in patients with IC compared with healthy controls (ICAM: 316.7ng/ml versus 207.7ng/ml, $P<0.001$ and VCAM: 485.1ng/ml versus 464.4ng/ml, $P<0.001$) (203). Both markers increased significantly following exercise (ICAM: 316.7ng/ml to 420.8ng/ml in patients with IC and 207.7ng/ml to 262.8ng/ml in healthy controls, $P<0.001$ and VCAM: 485.1ng/ml to 576.2ng/ml in patients with IC and 464.4ng/ml to 544.2ng/ml in healthy controls, $P<0.001$), although the rise was

more significant in patients with IC (203). Brevetti et al (207) reported similar results for both VCAM and ICAM in patients with IC who walked to MWD (ICAM: 285ng/ml to 317ng/ml, $P<0.01$ and VCAM 671ng/ml to 751ng/ml, $P<0.05$). Consistent with the cytokine levels in Andreozzi et al (202) (*See section 1.3.4.2.1* Contribution of IRI to systemic inflammatory burden*)*, Brevetti et al (207) demonstrated that levels of these CAM's had markedly subsided to near normal levels after 30 minutes of recovery from exercise. Furthermore, brachial artery FMD, the widely utilised non-invasive marker of endothelial function, has also been found to be acutely and significantly impaired in response to an isolated bout of aerobic exercise to onset of claudication pain (208, 209) and MWD (210). Joras et al (210) demonstrated this acute decline in endothelial function to return to resting levels after 4 hours of recovery.

1.3.4.2.3 Mechanism of endothelial activation following exercise in IC

It is worthwhile to consider the mechanism of endothelial activation that leads to up-regulation of CAM's and decline in FMD in patients with IC following exercise. Ischaemia-reperfusion injury and associated increased levels of inflammatory cytokines undoubtedly play a role due to the oxidative stress and associated breakdown of NO (194). Furthermore, the cytokines released by skeletal muscle as part of the physiological response to exercise, are also likely to contribute to levels of oxidative stress immediately following exercise and would explain the acute impairment of endothelial function observed in healthy individuals after a bout of exercise (211). Additionally, independent of IRI, flow dynamics are likely to be impaired in patients with PAD due to the turbulent flow induced by atherosclerotic lesions. During exercise, increased metabolic requirements lead to augmentation of cardiac output and subsequent increased flow volume through lesions that are unable to dilate in response to shear stress. The resultant worsening of turbulent flow is likely to further impair shear stress induced production of NO, thus causing an acute deterioration in endothelial

function and an associated up-regulation of CAM expression, ultimately leading to a pro-inflammatory response, with the potential to further worsen the degree of ED.

It is likely therefore that alterations in flow dynamics act in synergy with IRI to account for the endothelial activation and pro-inflammatory state that occurs in response to an acute bout of exercise in patients with IC. Such an interaction may also be responsible for the evidence demonstrating a chronic increase in baseline levels of cytokines such as IL-6 and TNF-α in patients with PAD (202, 203). This may represent a cumulative effect of the acute detrimental effects induced by exercise in patients with IC.

1.3.4.3 Significance of a pro-inflammatory phenotype

The clinical implications of a pro-inflammatory phenotype must not be understated. A futile cycle exists in which a pro-inflammatory state leads to further activation of a dysfunctional endothelium which in turn further precipitates a pro-inflammatory state (54). More specifically, certain pro-inflammatory cytokines have themselves been identified as markers of disease severity and progression. Interleukin-6 is not only a marker of the acute inflammatory response but is also associated with plaque instability and has therefore been deemed a pathophysiological feature related with progression of disease and therefore cardiovascular risk (212). Demonstrated impairments in lower limb functioning among patients with IC, as measured by fast-paced four metre walking velocity, also correlate with IL-6 levels (213). Interleukin-1 and TNF-α have also been shown to play a critical role in the pathogenic mechanisms leading to vascular inflammation and subsequent atherosclerosis (214, 215). To highlight this, separate studies using mice that are knockout for the IL-1 (216) and TNF-α genes (214) respectively have demonstrated a marked decrease in the severity of laboratory induced atherosclerosis.

1.3.4.4 Markers of systemic inflammatory burden in PAD

Although the inflammatory signature of PAD is complex and multi-factorial, comprising pro- and anti-inflammatory cytokines, acute phase reactants (eg CRP), markers of macrophage activation (eg neopterin) and the inflammatory cells (eg neutrophils) and their products (eg NE), two non-cytokine markers of systemic inflammatory burden that are referred to in this thesis are homocysteine and CRP

1.3.4.4.1 Homocysteine

Homocysteine is widely recognised as a risk factor for atherosclerotic disease, but its role as an inflammatory marker is often under-stated and worthy of consideration. It is a non-protein amino acid resulting from the demethylation of the essential amino acid methionine (217). Hyperhomocysteinemia has been linked to vascular disease since the early 1960's when children with mental retardation, accelerated growth and propensity to arterial and venous thrombosis were found to have homocysteinuria (217). An emerging pattern of atherosclerosis was detected in these patients and it was concluded that genetic defects in homocysteine metabolism and associated homocysteinuria was responsible for these vascular lesions (217).

Endothelial vascular injury and atherosclerosis was subsequently demonstrated through intravenous infusion of Hcy in an animal model (218).

Several studies followed this, reporting the association between Hcy concentrations and vascular disease and more recent large scale meta-analyses have supported these findings (219). High values have also been proven to predict the failure of vascular intervention and more rapid progression of CAD and PAD (220). More recently, circulating levels of homocysteine have been proposed as an inflammatory marker, largely due to its ability to promote oxidative stress (221).

Hyperhomocysteinemia is prevalent in diseases such as inflammatory bowel disease and rheumatoid arthritis and anti-inflammatory medications have demonstrated homocysteine lowering effects (221). Significantly, an intensive, acute bout of endurance exercise in healthy individuals induced a significant increase in serum homocysteine levels (222), which could be explained by the known pro-inflammatory effect of acute exercise. Consistent with other inflammatory markers, longer term exercise training among healthy adults promotes an adaptive lowering of homocysteine levels (223). Despite this, evidence provided by a rat model of renal IRI has shown an increase in homocysteine levels associated with reduced activity of cystathionine-ß-Synthase, a key enzyme in homocysteine metabolism (224). Whether or not such findings are translated to exercising patients with IC remains to be seen.

1.3.4.4.2 C-reactive protein (CRP)

C-Reactive Protein is an acute phase protein known to play a role in a number of pro-inflammatory immune-modulatory pathways including complement activation, activation of leukocyte chemotaxis and upregulation of pro-inflammatory cytokine synthesis and release (192). Its non-specific nature means that it can be used to represent the systemic inflammatory burden of an individual. Levels of CRP are known to be increased in patients with PAD and other atherosclerotic diseases (225). While the exact role of CRP in the initiation and progression of such a disease state is unclear, it has been localised in atherosclerotic lesions and it is established that raised levels of CRP are associated with a worse prognosis for cardiovascular disease (225).

There is extensive evidence to suggest that CRP levels are acutely increased following vigorous exercise in healthy individuals. Despite this, all studies investigating the response of CRP to acute bouts of exercise in patients with IC have demonstrated that CRP levels are unaffected (151, 192, 204). One could speculate that this may be reflective of the fact that patients with IC are unable to exercise at an intensity sufficient to promote CRP release as a

result of muscle damage, the mechanism proposed to account for CRP rise following exercise in healthy individuals. Furthermore, it may be that there is a limited role for CRP in IRI in patients with IC, although this seems unlikely given the evidence implicating CRP with myocardial IRI (226). Most likely is the marked inter-individual variability of CRP noted in each relevant study, masking any change which may have otherwise been detected.

1.3.4.4.3 Other pro-inflammatory markers

It should be mentioned that there are numerous other markers of inflammation and endothelial activation that have been used to demonstrate an acute pro-inflammatory response of patients with IC to exercise. These include thromboxane (marker of platelet activation), lipid peroxides (oxidative free radicals), serum amyloid A (SAA, an acute phase reactant) and thrombomodulin (an endothelial cell membrane glycoprotein released with cell damage) (227).

1.3.4.5 Impact of supervised exercise training on inflammatory burden in IC

Sustained exercise training seems to modify the pro-inflammatory response and augment an anti-inflammatory response in healthy individuals (*See section 1.3.4.1* Pro versus anti-inflammatory response*)*. A key question is therefore does a sustained exercise program in patients with IC induce the same adaptive anti-inflammatory response as has been demonstrated in healthy individuals, or is exercise and the associated IRI and endothelial activation precipitating a worsening of systemic inflammatory burden in these patients with a subsequent detrimental impact on long-term health outcomes?

To date, there is limited work to address such a question. Significantly, only one (228) of the four studies (158, 228-230) identified to assess the impact of supervised exercise training on inflammatory burden has implemented a treadmill based supervised training regimen consistent with the current consensus guidelines. Furthermore, in two (158, 229) of the four

studies the duration of the intervention ranges from 2-4 weeks, well short of the recommended 3-6 months.

In a review article, Tisi et al (227) have cited their own work (229) to suggest that supervised exercise training attenuates the inflammatory response to acute exercise in patients with IC. Such work assessed the impact of a 12 month exercise program on levels of the acute phase inflammatory proteins CRP and SAA as well as fibrinogen (a marker for onset and progression of PAD) and urinary albumin-creatinine ratio (ACR, which is reflective of vascular endothelial permeability). Levels of CRP (5.3mg/L to 4.4mg/L, $P<0.05$) and SAA (70mg/L to 20mg/L, $P<0.01$) were significantly reduced after 3 and 6 months of exercise respectively, however, CRP had increased again to baseline levels after 6 months (229). Fibrinogen levels remained unchanged throughout the training. A significant post-exercise increase was detected in ACR (exact values not stated) and although a trend was evident demonstrating an attenuation of such an increase throughout the program, this never became significant (229). A limitation of this study is the fact that only 4 weeks of the 12 month study duration was supervised treadmill based exercise, with the remainder being home-based walking, the frequency and intensity of which was recorded as reported by participants to assess compliance. Self-reported exercise sessions certainly trended towards lower values from 3-12 months, however, this was non-significant. Despite this, the accuracy of self-reporting over such a long duration is questionable and brings into question the validity of data beyond 3 months. Furthermore, the results presented by Tisi et al (229)are certainly not conclusive and his conclusion that *"the concern that exercise has adverse systemic effects therefore seems to be unjustified"* is itself a comment that is perhaps not justifiable given the available evidence he has presented.

In the most recent article published, results from Schlager et al (228) in some way support the conclusions of Tisi et al (229). In this case however, at various time points during 6 months

of supervised treadmill based training, resting levels of IL-6, CRP and fibrinogen remained unaffected by exercise.

Two studies of shorter duration also failed to demonstrate either a pro or anti-inflammatory response to an exercise program (158, 230).

In one arm of a study designed to assess the benefits of iloprost (a prostacyclin analogue with potent vasodilatory effects) versus supervised exercise training on expression of CAM's and markers of platelet function, Arosio et al (158) randomised 12 patients with IC to two weeks of an intensive daily exercise program, consisting of treadmill training, cycling and dynamic lower limb resistance exercises. Such exercise did not significantly alter levels of CAM's (specifically VCAM). Although not anti-inflammatory, a mild anti-thrombotic effect of exercise was demonstrated through a reduction in ex-vivo platelet adhesion to fibrinogen (a substrate for platelet adhesion) and the down-regulated expression of fibrinogen receptor on platelets.

Nawaz et al (230) subjected 52 patients with IC to either upper or lower limb supervised cranking exercises for a period of 6 weeks and investigated levels of neutrophil (CD11b, CD66b) and endothelial (E-selectin) activation markers at various time points. While an acute bout of lower limb cranking exercise significantly increased expression of CD11b and CD66b from baseline (exact values not provided), resting expression of these markers and E-selectin was unaffected by the training program. When interpreting such findings, it is important to consider that the method of exercise (ie cranking) used in this study was chosen so not to induce claudication pain and potentially minimise the inflammatory response associated with IRI.

The lack of any real pro or anti-inflammatory response in these studies is somewhat surprising given the known pro-inflammatory response to acute bouts of exercise in both

patients with IC and healthy controls. Such findings could be explained by a combination of heterogeneous factors that were observed across the four studies including duration and type of exercise, as well as the choice of inflammatory markers. It is possible that the identified markers simply may not be reflective of the chronic inflammatory response associated with repeated bouts of exercise.

1.3.4.6 Impact of supervised exercise training on endothelial function in IC

Despite the fact that improvement in endothelial function has been proposed as a key mechanisms by which SET can facilitate clinical improvement in walking performance in patients with IC, very little attention has been afforded to this in the literature. In 2009, McDermott and colleagues (122) assessed the endothelial function of 37 patients with IC before and after undertaking a 6 month treadmill based exercise program. Pain free walking time and MWT improved significantly however, endothelial function, as represented by FMD was unchanged. Prior to this, Brendle et al (231), in a non-randomised, non-controlled trial with a 6 month supervised treadmill intervention reported contrasting results, with an observed significant improvement in FMD (4.81% to 7.97%, $P<0.005$). Similar results were reported by Andreozzi et al (232) who, in a prospective cohort study, reported a significant improvement in resting FMD after a 6 week treadmill based exercise intervention (7.6% to 10.3%, $P<0.01$).

The systemic cardiovascular adaptations associated with lower limb exercise are such that they can manifest as changes to the systemic state of endothelial function, as measured by FMD in the brachial artery. Despite this, a major limitation of all three studies is the fact that assessment of FMD was undertaken following upper arm (proximal) occlusion of the brachial artery. This has recently been shown to invoke both endothelium dependent and independent hyperaemic response, of which the relative contributions have not been quantified (233). It is possible therefore that treadmill based exercise training can augment the endothelium

independent hyperaemic response, rather than the true NO mediated endothelium dependent response (233).

In order to make any meaningful conclusions about the role of the endothelium in mediating response to treadmill based exercise in patients with IC, more evidence is required. This should focus specifically on the impact of supervised exercise training on endothelium dependent vasodilation, which can be achieved by occluding the arm distal to the segment of brachial artery being visualised for assessment of FMD.

Irrespective of the impact of SET on inflammatory status and endothelial function, perhaps the only way to accurately quantify the impact of SET on the systemic health of a patient is through the assessment of long-term health outcomes. Unfortunately, the length of follow-up required to identify such outcomes is challenging, largely due to difficulty with participant retention. Furthermore given that the sample size of most exercise based interventions is relatively small, a lack of statistical power may make meaningful conclusions difficult to reach. The 2008 Cochrane review (110) to determine the effects of exercise on IC highlights this problem by stating that mortality and amputation data were inconclusive and that no data were available to assess non-fatal cardiovascular events.

1.3.5 Does exercise modality influence response?

Independent of their assessment of the inflammatory response to SET, the four studies (158, 228-230) identified to explore the impact of SET on inflammatory burden (*See section 1.3.4.5* Impact of supervised exercise training on inflammatory burden in IC*)* reveal an interesting concept relevant to the improvement of walking performance, irrespective of the type of exercise regimen. While both Tisi et al (229) and Schlager et al (228) adopted a primarily walking based regimen, Nawaz et al (230) used upper and lower limb cranking exercises and Arosio et al (234) subjected patients to a combination of cycling, lower limb resistance and

treadmill training. All reported improvements in walking performance measured as either PFWD (234), MWD (228) or both (229, 230). Nawaz et al (230) even concluded that in the absence of any detectable rise in inflammatory biomarkers, such variations in exercise regimen may offer certain advantages over the currently recommended treadmill based regimens.

Such a concept has been adopted by several groups and there now exist exercise trials in patients with IC incorporating a range of modalities including polestriding (33) and stairmaster (235).

Of interest are those randomised trials that have assessed the benefit of treadmill based exercise compared to alternative exercise modalities with respect to walking performance.

1.3.5.1 Resistance training

Based on the observation that patients with PAD have a relative reduction in muscle strength associated with muscle atrophy (183), it has been proposed that resistance training in these patients may improve strength and subsequently functional performance and walking ability (183). Three studies (122, 123, 236) have assessed the superiority of treadmill based exercise compared with resistance training in patients with IC. Hiatt et al (123) reported that after 12 weeks of supervised exercise, lower limb resistance training significantly improved MWT (6.5mins to 11.8mins, $P<0.05$), however, this tended to be to a lesser extent than the improvement obtained from treadmill based training (9.6mins to 17.2mins, $P<0.05$) which also provided significant improvements in PFWT (3.3mins to 10.2mins, $P<0.05$). Contrasting results were reported by Ritti-Dias et al (236), whose methods were quite similar to those of Hiatt et al (123), with the exception that whole body resistance exercises were prescribed rather than just lower limb resistance training. Both PFWD and MWD improved significantly and comparably within strength training and treadmill training groups (strength training

group, PFWD: 358m to 504m and MWD: 618m to 775m, P<0.01, treadmill training group, PFWD: 342m to 469m and MWD: 572m to 721m, P<0.01) (236).

While it could be speculated from the above results that greater cardiovascular adaptation may be promoted by a whole body resistance training regimen, McDermott et al (122) employed a 6 month exercise program in which resistance training consisted of lower limb exercises only and achieved significant improvements in PFWT (3.2mins to 5.1mins, P<0.05) and MWT (7.2mins to 9.6mins, P<0.05).

Although not directly comparable to the benefit of treadmill training, further support for the role of resistance training is provided by McGuigan et al (237), whose randomised trial assessed the benefit of 6 months whole body resistance training with non-exercising controls. A significant 158% improvement in PFWD (actual values not recorded) was recorded in resistance trained patients, while there was no change in the control group (237). Helping to shed some light on the molecular mechanism for improvement in walking performance associated with resistance training, McGuigan et al (237) also obtained gastrocnemius muscle biopsies before and after the intervention. Analysis revealed adaptation of skeletal muscle in the form of increased capillary density and muscle fibre area after the intervention. In addition, an increase in the proportion of the more fatigue resistant type IIa fibres was noted, relative to the type IIb fibres with low levels of mitochondria and associated easy fatigability (237).

1.3.5.2 Combination regimens

Intermediate to both walking and resistance training are a host of trials in which the exercise intervention consisted of a combination of lower extremity aerobic activity ranging from cycling (116) and calf raises to dribbling a soccer ball (238). Of these, only one trial has been identified that was randomised to allow a comparison of lower extremity aerobic exercise and

the current gold standard of supervised treadmill based training (116). In this study, supervised cycling was the exercise of choice for a period of 6 weeks. While treadmill training significantly improved both PFWT (6.9mins to 10.1mins, $P<0.05$), walking performance in cycle-trained individuals was unaffected by the intervention (116).

Despite these results, within group improvement in walking performance has been demonstrated by numerous studies comparing lower extremity aerobic exercises with usual care controls (229, 230, 239, 240), percutaneous angioplasty (238, 241), upper limb aerobic exercise (242) or simply in prospective cohort studies (243, 244). Such study design however, does not enable determination of the superiority of such training regimens as compared with treadmill training alone. Furthermore, the lower extremity aerobic exercise intervention in many of these studies comprised a combination of treadmill and dynamic leg exercises (238, 244), treadmill, cycling and resistance exercises (243) or treadmill and resistance training (240). It is therefore impossible to identify the relative contribution of treadmill training alone versus that of the other modalities to the improvements in walking performance reported in these studies. Meaningful conclusions can therefore not be drawn from these data.

1.3.5.3 Upper-extremity aerobic exercise

More recently, an interesting new concept has evolved that suggests a role for upper extremity aerobic exercise in the treatment for IC. It has been proposed that unlike walking and lower limb aerobic exercise, upper extremity exercise does not induce IRI and associated pain, therefore enabling patients to achieve a higher exercise intensity with associated improvements in systemic cardiovascular conditioning and walking performance (121). Additional benefit may be derived through a mechanism conveniently termed a “transfer-effect”, in which exercise training in one muscle group can enhance the performance of other untrained muscle groups through a generalised systemic training effect (121) which may result in improved lower limb oxygen delivery (245).

Walker et al (242) first identified the potential of such a concept in 2000 when he randomised 52 patients to either upper or lower limb cranking exercises for 6 weeks and reported significant and comparable improvements in walking performance within both groups (upper limb group, 122% improvement in PFWD, lower limb group 93% improvement in PFWD, absolute values not stated). In support of these results is work from Zwierska et al (246), who used equivalent methods with a larger sample size (n=104) and longer duration (6 months) of intervention to support the results of Walker et al (242) (upper limb group, 51% improvement in PFWD, lower limb group 57% improvement in PFWD, absolute values not stated) .

Subsequently, Treat-Jacobsen et al (121) investigated the efficacy of arm cranking versus the gold-standard supervised treadmill training, as well as a usual care control group and a combination of treadmill training and arm cranking exercise. After a 12 week intervention MWD improved significantly in all three exercise groups compared with the control group, with no significant differences observed between exercise groups (change in MWD: (a) treadmill group 295m, (b) combination group 217m, (c) arm cranking group 182m, (d) control group 45m) (121). Similar results were observed for PFWD, however, baseline PFWD was found to have a significant effect on these results and when adjusted for, only the arm-ergometry group recorded a statistically significant improvement in PFWD (change in PFWD: 90m, P=0.03) (121). Interestingly, the improvement in the combination group was similar to that of the other two exercise groups and failed to demonstrate an additive effect that may have been expected given the independent benefits that occurred with treadmill training and arm cranking. Treat-Jacobsen et al (121) suggested that improvement may be due to total volume of exercise, which in this case was similar across each group, rather than the modality itself. Alternatively, there may exist a maximum benefit of improvement in walking performance that can be conferred by exercise, as determined by the physiological

response of an individual (eg maximum up or down-regulation of relevant gene expression). Whether or not a biomarker exists to predict such a level of benefit remains to be seen.

The small sample size (n=8 or n=9) in each group is certainly a limitation of this study from Treat-Jacobsen et al (121), however, with such favourable preliminary results, further work is definitely warranted. The need for further work in this area is also highlighted by Parmenter et al (111), whose 2011 review discussing alternative exercise prescription as a treatment for IC, concluded that "*additional studies of high quality are required to validate these alternative prescriptions and their efficacy relative to walking"*. A Cochrane review protocol has also recently been proposed to investigate such a topic (141).

Although Treat-Jacobsen et al (121) have superficially explored such a concept, further consideration should also be given to the potential additive benefit that may be gained by combining walking with other types of exercise that have been shown to have independent effects, for example resistance training.

Importantly, performance of such alternative exercise modalities may limit the exposure of patients with IC to IRI, thus avoiding the potential detrimental effects of IRI such as worsening of endothelial function and precipitation of a pro-inflammatory response. Therefore, when discussing what the most appropriate form of exercise is for patients with IC, the systemic impact of such a program also warrants critical thinking, with a view to ensuring that these patients are not being placed at an increased risk of future cardiovascular events.

1.3.6 Nutritional considerations in exercising PAD patients

An often overlooked entity which is vital to the systemic well-being of an individual is that of nutritional status. Maintenance of nutritional status is dependent on the intake of a diet sufficient to meet or exceed the needs of an individual required to preserve body composition

and function (247). Such requirements may be increased by processes resulting in decreased intake, increased utilisation or altered utilisation and if not compensated for, loss of body mass occurs (247).

Exercise is one such process associated with increased metabolic demands and utilisation. This is a particularly important consideration for patients with IC who are already known to be nutritionally vulnerable (248). Patients with PAD have relative muscle atrophy when compared to controls (248), while obesity is an established risk factor for PAD (7), often masking underlying muscle wasting resulting in a state known as sarcopenic obesity (defined as an increase in fat mass and decrease in lean mass) (249). Whether or not the relationship between nutritional status and PAD is associated with dietary intake or related to physiological adaptations to the disease state is unclear, however, it is likely a combination of both. In a large scale population based study, Lane et al reported that dietary intake of higher levels of nutrients including vitamins and fibre may confer a protective effect against PAD (250). Providing further evidence to support this theory is work from Gardner et al (251) who in a trial of 46 patients with IC demonstrated high intake of pro-atherogenic foods including saturated fat, sodium and cholesterol and low intake of potentially anti-inflammatory and anti-oxidative foods such as fibre, vitamin E and folate compared with recommended daily dietary intake.

While dietary protein was unreported in Lane et al (250), in the aforementioned study from Gardner et al (251), 67% of patients with IC either reached or exceeded the recommendations for protein intake. This implies that another mechanism(s) may be responsible for the documented muscle atrophy occurring in these patients. It is established that irreversible muscle cell death and subsequent muscle atrophy is a consequence of IRI. The calpain system of proteolytic enzymes may play a mechanistic role in such a response (*See section 1.3.3.2.3* Calpain system*)*. While exercise training of any modality would be expected to improve

skeletal muscle mass and the atrophied state of the muscle groups involved, in patients with IC it would seem reasonable to speculate that increasing the frequency of exposure to IRI, such as in treadmill based supervised exercise training, may serve to worsen the extent of muscle atrophy and have a detrimental impact on functional ability. Such a concept has not previously been assessed and reported in the literature, although in a rat model ischaemia induced muscle atrophy was demonstrated to be more severe in exercise-trained rats than untrained rats (182). A review article from Wolfe (252) highlights the importance of muscle mass in optimising health outcomes. The central role played by muscle in whole body protein metabolism is particularly important in response to physiological and pathological stress. Preservation of muscle mass is an important determinant of survival in chronic disease states such as cancer and cardiac failure. Ability to perform activities of daily living (ADL) and therefore maintain quality of life is also critically dependent on muscle mass (252).

Maintenance of a large muscle mass associated with a high rate of protein turnover can also contribute to the prevention of obesity (252). Muscle protein synthesis is dependent on muscle mass and energy expenditure is required to incorporate amino acids into protein. Changes in muscle metabolism are therefore reflected in the daily resting energy expenditure (REE) of an individual (252). The term REE represents the amount of calories utilised by an individual in an inactive state over a 24 hour period (252). The gold standard method of evaluation is the use of doubly-labelled water, which involves the ingestion of a quantity of water labelled with a known concentration of naturally occurring stable isotopes of hydrogen and oxygen (253). As energy is expended in the body, carbon dioxide and water are produced and the differences between the isotope elimination rates are used to calculate REE, however, the cost of materials and requirement of a mass spectrometer to analyse isotope concentrations prohibits the routine clinical use of such a technique (253). Alternatively, a cheaper and more accessible technique is known as indirect calorimetry. This provides an

accurate estimate of REE by measuring production of carbon dioxide and consumption of oxygen using a gas-exchange circuit (253).

Skeletal muscle mass is the largest contributor to REE (254) and given the relative muscle atrophy that has been demonstrated in patients with PAD, it would seem reasonable to suggest that REE may be reduced in these patients. Such a theory was confirmed by Gardner et al (255), who used indirect calorimetry to demonstrate that patients with IC have lower REE than age-matched, healthy controls. In a later article, Gardner and Montgomery (256) classified patients according to disease severity using the Rutherford classification and showed that REE was lower in patients with more advanced disease. If these findings are coupled with the sedentary lifestyle of many PAD patients, an energy imbalance is likely to exist in which input is greater than output and fat accumulation ensues, potentially leading to the nutritional state of sarcopenic obesity. A futile cycle may then be entered into with further work from Gardner et al (257) suggesting that abdominal obesity is predictive of disease progression.

Exercise in healthy individuals is known to increase REE due largely to increases in muscle mass (258), although studies involving high intensity exercise interventions have reported reductions in REE, in spite of increases in muscle mass (258). The reason for this is unclear but may reflect a skeletal muscle metabolic adaptation to enhance efficient energy utilisation during vigorous activity (258).

There are currently no studies reporting the impact of SET on REE in patients with IC. In fact, the influence of SET on the nutritional status of patients with IC has been poorly addressed in the literature, despite the consequences associated with increasing metabolic demands through high-intensity exercise without the concurrent administration of adequate nutrition in this already nutritionally vulnerable group. Evidence exists to suggest that

combined nutrition and exercise interventions in the rehabilitation setting are preferable, with those receiving exercise alone demonstrating greater declines in nutritional status and physical health (259, 260). Further to this, there is evidence to suggest that the timing and composition of nutritional support, in particular supplementary protein, is critical in achieving optimal outcomes for exercise interventions (261).

1.3.7 Summary

Intermittent claudication is a common presentation of PAD. Regular physical activity can improve symptomatology and prognosis in those with chronic disease and current consensus guidelines recommend that supervised treadmill based exercise training should be made available as a first line treatment for adults with IC. Such a recommendation is based on evidence demonstrating improvement in walking performance following supervised exercise training of sufficient intensity to induce moderate claudication pain. A careful analysis of such evidence has revealed a marked variability in reported response to exercise which may be attributable to heterogeneity of study design and assessment techniques. While the evidence likely still represents a beneficial effect of SET on the walking performance in patients with IC, the mechanism for such improvement remains unclear and findings must be interpreted with caution. Furthermore, the large variability in response to exercise makes identification of those likely to benefit from exercise training important. Despite this, there is insufficient evidence in the current literature to enable such predictions to be made.

Literature is also scant in relation to the long-term health outcomes of patients with IC undertaking supervised exercise programs. Adverse consequences of SET are possible and warrant exploration. In particular, the potential for muscle atrophy and the link between IRI, systemic inflammatory burden and ED, may prove that treadmill based exercise training is detrimental to the long term health of patients with IC. Although the prescription of an exercise program to patients with IC is focused on achieving improvement in walking

performance, this should not come at the expense of physiological changes that are likely to negatively impact on the health of the patient.

More recently, alternative exercise regimens have been proposed, with weak evidence demonstrating improvements in walking performance equivalent to the traditional treadmill based regimens. The ability of such regimens to limit exposure to IRI may prove beneficial to the long term health outcomes of patients with IC and further large scale studies are warranted.

1.4 Aims, research questions and hypothesis

The introduction to this thesis has highlighted a number of issues around the appropriate prescription of SET for patients with IC. In particular, the need to prescribe exercise programs which can improve walking performance in patients with IC without compromising long-term health outcomes.

1.4.1 Aims

The aims of this thesis, therefore, are to determine, in adults with intermittent claudication, whether 12 weeks of supervised exercise training with interval treadmill and lower limb resistance training produces more clinically and statistically significant improvements than a supervised treadmill exercise program alone and is therefore superior in terms of walking performance, quality of life and physiological markers of long-term health outcomes.

1.4.2 Research Questions

This thesis will present and discuss the findings from research undertaken by the candidate to answer the following research questions with a view towards addressing the principal aims (*See section 1.4.1* Aims*).*

Research question 1 (RQ1): Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant improvement in **PFWD** and **Quality of Life (QoL)** compared to an exercise regimen consisting of treadmill training alone in patients with IC?

Research question 2 (RQ2): Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant improvement in non-invasive markers of **endothelial function** (FMD, RHI, NO and ADMA) compared to an exercise regimen consisting of treadmill training alone in patients with IC?

Research question 3 (RQ3): Does 12 weeks of SET consisting of interval treadmill and lower limb resistance training impact differently **on systemic inflammatory response** and burden than an exercise regimen consisting of treadmill training alone in patients with IC?

Research question 4 (RQ4): Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant gains in **skeletal muscle mass** compared to an exercise regimen consisting of treadmill training alone in patients with IC and if so, is this associated with changes in **calpain** and **calpastatin** activity, **dietary intake** and **resting energy expenditure**?

1.4.3 Hypotheses

The hypotheses related to the above Aims and Research Questions are:

1. Interval treadmill and lower limb resistance training will lead to greater improvements in walking performance and therefore QoL than treadmill training alone due to the additive effects of mechanisms responsible for improvements in both treadmill and resistance training.

2. Interval treadmill and lower limb resistance training will result in less ischaemia-reperfusion injury and therefore a reduction in the systemic inflammatory response and greater improvement in endothelial function relative to treadmill training alone.

3. Interval treadmill and lower limb resistance training will result in a greater gain in muscle mass compared to treadmill training alone. This will be reflected in proteomic analysis with a greater reduction in calpain activity and a compensatory gain in calpastatin activity expected to be observed with interval treadmill and lower limb resistance training. As well, in conjunction with a gain in muscle mass, a greater increase in resting energy expenditure and improvement in dietary composition will be observed with interval treadmill and lower limb resistance training compared with treadmill training alone.

CHAPTER 2: METHODOLOGY

2.1 Study Design and Setting

This study was an RCT of 35 adults (26 Men, 9 Women) with IC, recruited from the Vascular Surgery Claudication Clinic conducted within the region of the Southern Adelaide Health Service. Participants were randomly allocated to: a) 12 week treadmill based SET with medical management of risk factors *or* b) 12 week combination (lower limb resistance and interval treadmill training) based SET with medical management of risk factors. Prior to commencement, the trial was registered with the United States Clinical Trials Registry and was allocated trial number *NCT 01871779*.

2.2 Study Population and Eligibility

All patients attending Vascular Surgery Outpatient Clinics within the region of Southern Adelaide Health Service who were diagnosed with IC by a consultant Vascular Surgeon or by referral from General Practitioner were referred to the Claudication Clinic (conducted by the candidate) based at the Repatriation General Hospital. The Claudication Clinic is an official outpatient service provided by the Southern Adelaide Health Service which specialises in the assessment and management of patients with IC. All screening and recruitment of eligible participants occurred within this clinic.

Upon presentation to the Claudication Clinic, resting ABPI's were performed on each patient, to confirm a diagnosis of PAD.

Ankle Brachial Pressure Indices were obtained with the patient supine on a bed using standard Doppler pressure technique (262, 263). A blood pressure cuff, sphygmomanometer and continuous–wave Doppler (Vista ABI L450VA, Summit Doppler, Golden, Colorado) were used to measure the systolic blood pressure (in mmHg) in both brachial arteries and in

the posterior tibial and dorsalis pedis arteries in both ankles. The ABPI for each ankle artery was the systolic blood pressure of that artery divided by the higher of the two brachial artery pressures. Patients with an ABPI<0.9 in any ankle artery were considered to have evidence of established PAD and were eligible for inclusion in the study.

Patients then underwent a clinical assessment to ascertain the symptoms related to their PAD and any previous vascular surgical intervention that they may have undergone. Patients were not eligible if they: (1) experienced lower limb ischaemic rest pain, (2) had clinical evidence of tissue loss such as ulcers or necrotic lesions, (3) had recently (<12 months) undergone peripheral vascular intervention (open surgery or endovascular), (4) suffered from pre-existing cardio-respiratory morbidities limiting exercise capacity, or (5) were deemed to be not competent of providing informed consent. Patients who described calf pain while walking that is relieved by rest were diagnosed as having IC and were eligible for further sonographic assessment to characterise their anatomical distribution of PAD. To further improve homogeneity of the group, patients with sonographic evidence of aorto-iliac disease were excluded, leaving only patients with confirmed infra-inguinal PAD as eligible for inclusion and participation in the SET.

Regardless of whether or not patients attending the Claudication Clinic were eligible for inclusion in the study, counselling was provided with respect to cardiovascular risk factor and lifestyle modification (smoking cessation, regular exercise, healthy diet, weight loss) and review of medications was undertaken to ensure that unless contraindicated, all patients were taking recommended pharmacotherapy (anti-platelet and lipid-lowering therapy and anti-hypertensive medication) to prevent disease progression and cardiovascular events associated with atherosclerosis.

2.3 Procedure for informed consent

The Southern Adelaide Clinical Research Ethics Committee approved all study procedures. All patients who met inclusion criteria were asked to participate in the study and provided with an information sheet to outline the background and rationale for the study, the commitment required during the study and the scheduled visits for outcome assessments and procedures to be undertaken. The two SET programs were described to patients and they were informed that allocation to the SET would be undertaken randomly. In addition, the candidate provided a verbal description of the study procedures and patients were given the opportunity to ask questions or raise any concerns that they may have about the study. For all patients who agreed to participate, written, informed consent was subsequently obtained.

2.4 Randomisation to study interventions

Randomisation was performed by the candidate after informed consent and baseline assessment had been undertaken. It was performed with a computer based random number generator (Excel 2010; Microsoft, Seattle, Washington, USA) using a 1:1 allocation ratio for block sizes which represented the number of participants recruited within each 3 monthly interval.

2.4.1 Blinding

It was not possible to blind either participants or the investigator (PhD candidate) from the trial allocation. Importantly, the assessor of each outcome measure was blinded to allocation. In the case of walking performance (PFWD and 6MWD), the assessor was a senior physiotherapist who had not been involved in the delivery of the intervention. Non-invasive markers of endothelial function were assessed by a trained sonographer with experience in FMD and RH-PAT techniques. Data pertaining to anthropometry, body composition and dietary intake was collected and assessed by an Advanced Accredited Practising Dietitian.

Serum samples were collected by the PhD candidate but assessed for inflammatory markers by trained laboratory staff. Proteomic assessment of muscle biopsies and assessment of serum biomarkers of endothelial function were performed by the candidate, however, at the time of collection, samples were de-identified to enable assessment to take place in a blinded manner.

2.4.2 Sample Size

For the primary outcome of PFWD, sample size was estimated based on baseline PFWD reported by Gardner et al (172m) (114)and the mean improvement in PFWD following SET suggested by the recent Cochrane review (82.2m) (110), approximately a 30% improvement. Data from McGuigan et al (237) suggested that improvement in PFWD following 24 weeks of resistance based SET was 158%, an additive effect of resistance based SET of 128% when combined with the approximate 30% improvement from SET in the Cochrane review. Given the intervention was 12 weeks, the expected effect size was halved to 64% (110m), a conservative SD of 75m was utilised (alpha = 0.05 and beta = 0.8) and the total sample size required was calculated to be n=28 (14 per group).

2.5 Design and delivery of the intervention

2.5.1 Structured Exercise Program

Consistent with recommendations from International Consensus Guidelines (26), The SET ran over 12 weeks and consisted of two 60 minute supervised exercise sessions per week. The program took place in the Repatriation General Hospital Rehabilitation Gymnasium and was supervised by both the candidate and a senior clinical physiotherapist with experience in administering exercise interventions for patients with cardio-respiratory disease. Prior to the commencement of the SET, all patients were advised to maintain their baseline level of daily activity for the 12 week duration of the SET.

2.5.1.1 Treadmill only allocation

Participants in the treadmill only group were advised to walk beyond the onset of claudication pain until pain became unbearable. They were then advised to rest until the resolution of pain and repeat the cycle for the duration of the session (60 minutes). Participants were instructed to complete as many cycles as possible during the session. Initial treadmill speed was determined by distance covered in the baseline 6MWT using the equation: speed = distance/time. If the participant progressed to the point where they did not experience symptoms within ten minutes of walking, the pace or gradient of walking was increased by 10% for intervals of increasing duration. In this way, an exercise stimulus was provided, designed to increase performance in accordance with the participants symptoms. Such a design is consistent with current Level A evidence suggesting that the most effective SET employs treadmill walking that is of sufficient intensity to induce claudication, followed by rest over the course of a 30-60 minute session (7).

2.5.1.2 Combination exercise allocation

Participants in the combination based SET group were educated to undertake three sets of 8-12 repetitions of hamstring curls, seated calf press, leg press, knee extension and hip abduction/adduction. For each exercise, participants started at the minimum level of resistance and were encouraged to increase resistance by trying to achieve 12 repetitions rather than the minimum of 8. Once this was achieved, the resistance increased by 5% and participants were again asked to try to progress to three sets of 12. Following completion of resistance exercises, participants were asked to walk on the treadmill until the onset of claudication pain only. They were then advised to rest until the resolution of pain before returning to again start resistance exercises. This cycle was repeated for the duration of each 60 minute session. Participants were instructed to complete as many cycles as possible during

the session. Initial walking speed and graded increases were determined as described in *section 2.5.1.1* Treadmill only allocation.

The design of the SET for this group was implemented to limit the duration of IRI relative to the treadmill only group who exercised to the extreme of claudication pain. It was predicted that this had the potential to limit the systemic inflammatory response, which coupled with the added benefits of resistance training (improved muscle mass/strength) would be likely to produce more clinically and statistically significant changes than treadmill only exercise with respect to the outcome measures proposed within this study.

2.5.2 Subjective assessment of exercise intensity

In order to compare the level of intensity induced by both the treadmill and combination training programs, patients were asked to quantify their perceived level of exercise intensity at both the commencement and completion of the SET. Borg's rating of perceived exertion scale has been shown to be a valid measure of exercise intensity and correlates with physiological measures such as heart rate and respiratory rate (264). The scale ranges from 6 (no exertion at all) to 20 (maximal exertion) and it is widely considered that a score of 12 to 14 is associated with a moderate level of exercise intensity.

2.5.3 Retention and compliance

Attendance at each exercise session was recorded and satisfactory completion of the SET was determined to be attendance at >80% of sessions. Participants who failed to attend a session without prior notice were contacted by the candidate to enquire as to their well-being and their intentions for the remainder of the program. Those who stated their intention to withdraw from the program were encouraged to provide a reason for doing so and were asked wherever possible to make themselves available for post-intervention outcome assessment for the purposes of intention to treat (ITT) analysis.

2.5.4 Concurrent educational lecture series

To supplement the SET and risk factor/lifestyle modification advice provided by the candidate in the Claudication Clinic, patients were also asked to attend several educational lectures provided by experienced Allied Health staff who work with patients with PAD. These lectures were designed to raise awareness and provide further insight into the contribution of risk factors and lifestyle choices to PAD, while at the same time providing advice and strategies on how to improve/modify such factors. The lecture series comprised of one half hour session per week for the first 6 weeks and topics included: Diet specific for PAD; The importance of healthy feet; Arterial Disease risk factors; The benefits of a walking program; Medication and arterial disease; Lifestyle and goal-setting. All patients from both groups attended the same sessions. *Appendix 1* shows the complete timetable.

2.6 Data collection

Baseline data collection commenced after consent had been obtained and prior to randomisation to exercise intervention. All data collection was performed by the candidate (or appropriately trained personnel, under the supervision of the candidate, as described in *section 2.4.1* Blinding*).*

2.6.1 Demographics and medical history

For all participants, clinical assessment in the Claudication Clinic involved collection of underlying comorbidities, current medications and smoking status. Information regarding gender and age was also readily available both from the participant and confirmed by hospital records.

2.6.2 Assessment of outcome measures

All primary and secondary outcomes were assessed within two weeks prior to the commencement of the SET and no later than two weeks following the completion of the 12 week SET.

2.6.2.1 Walking performance

While most studies investigating the impact of SET on PFWD have used a standardised treadmill test to assess walking distance (112, 116, 118, 120, 121, 123, 124, 127, 128, 135, 136, 236), concerns have been raised regarding the correlation between treadmill versus normal daily walking ability. It has been proposed that due to the different biomechanics required to ambulate on a treadmill, a degree of familiarity is required before optimal performance can be achieved (142). For this reason, the suggestion is that non-treadmill based walking assessment may be a more appropriate measure of walking distance (122).

The 6-minute walk test is a well validated, reproducible technique that is an objective measure of functional exercise capacity. It was therefore the measure of choice for this study (265).

The test required participants to be rested for 30 minutes before being asked to walk, self-paced along an indoor, flat, straight walkway, 20 metres long and marked at regular intervals. The test was conducted by a trained physiotherapist, who was blinded to allocation and participants were instructed to advise the physiotherapist immediately at the onset of claudication pain. This distance was recorded as PFWD. At this point, participants were asked to continue walking for the duration of the 6 minute test or until the pain became disabling. Participants whose pain required them to stop were allowed to rest until pain subsided before recommencing the walk test. The timer continued during any periods of rest.

The distance covered over the course of the 6 minutes was recorded as the 6 minute walking distance (6MWD).

2.6.2.2 Endothelial function

For all tests of endothelial function, participant preparation was consistent with recently published guidelines for the assessment of endothelial function using FMD (53). All testing was undertaken in the afternoon (between 1300 and 1700). Participants were requested to refrain from high fat foods, caffeine, tobacco and alcohol for at least eight hours prior to assessment and to avoid strenuous exercise on the morning of the test. Participants were asked to wait in a quiet, temperature controlled room for 20 minutes prior to the test.

2.6.2.2.1 Reactive hyperemia peripheral arterial tonometry (RH-PAT)

The reactive hyperemia index (RHI) was obtained using an EndoPAT peripheral arterial tonometry device (Itamar Medical Ltd, Caesarea, Israel). The blood pressure of each participant was obtained using the right arm with a standard blood pressure cuff and sphygmanometer and then the blood pressure cuff was placed, but not inflated, on the left upper arm. After an explanation of the test procedure the participant was seated comfortably at a desk and non-invasive pneumatic probes were attached according to the manufacturer's instructions. These were on bilateral index fingers unless the fingers of the participant were too large for the probes in which case a smaller finger was used. No talking was allowed, except for brief instructions required during the test. The EndoPAT device continuously recorded digital arterial pressures for the duration of the test, a total of 15 minutes. A 5 minute baseline period prior to occlusion was followed by 5 minutes of total brachial artery occlusion achieved with the cuff inflated to 250 mmHg (>50mmHg above systolic pressure), followed by a 5 minutes post occlusion period to measure the degree of reactive hyperaemia relative to the non-occluded right arm. The RHI was measured using the proprietary software on the EndoPAT device.

2.6.2.2.2 Flow mediated dilatation (FMD)

The FMD was obtained with the participant supine on a bed and performed directly following a 10 minute break after completing the RH-PAT test. The FMD technique was based on recently published consensus guidelines (53). The right arm was placed in a specially designed supporting cradle and a blood pressure cuff was placed around the forearm with the edge of the cuff 5cm distal to the medial epicondyle. As recommended by the consensus guidelines, the right arm was used to avoid confounding from the RH-PAT test which was performed on the left arm. A three point electrocardiogram (ECG) connected to the ultrasound machine allowed display of the ECG trace on the ultrasound image. An ultrasound system (SonoSite M-Turbo, SonoSite, Inc, Bothel, USA) with a high resolution linear array transducer (SonoSite HLF38x) with a broadband frequency range of 6 to 13 MHz was used to obtain high resolution images of the brachial artery. In particular the transducer position was adjusted until both the near and far arterial walls were well defined, the image was as free as possible of artefactual echoes and that the image size and gain were optimised. The transducer was placed in a specially designed stereotactic stand that allowed free movement while locating the brachial artery and then fixation of the transducer in any required position on the upper arm to allow constant imaging of the brachial artery over a prolonged period of time. A 30 second cine clip was obtained prior to cuff inflation to allow a pre-inflation brachial artery diameter to be obtained. The cuff was then inflated to 250mmHg to achieve total brachial artery occlusion. Post cuff deflation, 60 second cine clips of the brachial artery were obtained starting at 15, 80 and 145 seconds to ensure that artery diameters could be measured at 15 second intervals to at least three minutes after post occlusion. Maximal dilatory response was expected to occur during this time (53, 266).

The cine clips were transferred to a Picture Archiving and Communication System (GE Centricity 3.0.3, GE Healthcare Integrated IT Solutions, Barrington, IL, USA) for permanent

storage using the Digital Imaging and Communications in Medicine 3.0 standard format. The files were then extracted from the Patient Archiving and Communication System and transferred to a computer workstation for analysis. Specific automated software (Brachial Artery Analyser, MIA-LLC, Coralville, USA) was used to measure the brachial artery diameter pre occlusion and at 15 second intervals throughout the post-occlusion period for a total of 205 seconds (the time beyond which consensus guidelines suggest that further FMD is not expected to occur) This software utilizes edge detection and wall tracking algorithms to automatically measure the luminal diameter within a specified region of interest. This technology has been shown to improve the validity of FMD measurements compared to manual techniques (53, 267). All measurements were obtained in peak systole (using the displayed ECG trace) and were recorded as the average of three measurements obtained from different cardiac cycles, all within five seconds of each other. A pre-occlusion diameter was obtained and then diameters were obtained at 15 second intervals throughout the post occlusion period.

The FMD was calculated by obtaining the percentage increase in diameter of the brachial artery and the absolute maximum diameter. The maximum percentage increase was calculated from pre-occlusion diameter and the maximum post occlusion diameter as per standard FMD technique (53). The time from cuff deflation to maximum diameter was also recorded.

2.6.2.2.3 Serum biomarkers of endothelial function (NO and ADMA)

Using standard venepuncture technique, blood was collected into two 8ml lithium heparin Vacuette tubes. Samples were immediately placed on ice, before being spun at 4,500 rpm for 7 minutes at 20°C in an eppendorf Centrifuge 5702 (Eppendorf AG, Hamburg, Germany). Serum from each Vacuette tube was then selectively transferred into 2.0ml RNase and DNase free graduated free standing screw cap microtubes (Thermo Fisher Scientific Australia,

Scoresby, Victoria), before being transferred immediately for storage at -80°C in a Model 700 series ultra-low temperature freezer (Thermo Fisher Scientific Australia, Scoresby, Victoria).

On the morning of analysis, stored samples were allowed to thaw to room temperature, before levels of NO and ADMA were determined by ELISA. Standard 96 well Total Nitric Oxide Assay Kit (Thermo Fisher Scientific, Illinois, USA) and ADMA Human ELISA kit (Enzo Life Sciences, New York, USA) were used according to manufacturer's instructions. The NO assay used the enzyme nitrate reductase to convert nitrate to nitrite (both stable metabolites of NO). Nitrite was then detected as a coloured azo dye product of the Griess reaction that absorbs visible light at 540nm. Total NO contributed by nitrate and nitrite in a system is then measured as nitrite after converting all nitrate to nitrite. A Thermo Scientific Multiskan EX microplate photometer (Thermo Fisher Scientific Australia, Scoresby, Victoria) was used to read each plate and generate reports. Concentrations of NO and ADMA in each sample were subsequently recorded.

2.6.2.3 Systemic inflammatory response

2.6.2.3.1 Routine laboratory investigations

As part of routine vascular assessment and risk factor profiling within the Claudication Clinic, all participants were asked to undertake a commercial fasting blood test before and after the 12 week SET, to be performed and analysed at their local Institute of Medical and Veterinary Science (IMVS) laboratory. Requested analyses allowed for analysis of non-specific markers of systemic inflammatory response and included Full Blood Examination (from which levels of neutrophils, lymphocytes and subsequently neutrophil:lymphocyte ratio were extracted), as well as C-reactive protein (CRP) and plasma homocysteine.

2.6.2.3.2 Cytokine analysis

To enable assessment of the acute and chronic impact of exercise on inflammatory response and to determine whether or not such a response would be augmented throughout the SET, patients were asked to submit to venepuncture before exercise and immediately after exercise (prior to return to baseline heart rate) at the first and last session of the SET.

Blood samples were collected, processed and stored as described in *Section 2.6.2.2.3* Serum biomarkers of endothelial function (NO and ADMA). They were then transferred on dry ice via courier to the Australian Proteome Analysis Facility at Macquarie University, Sydney, NSW where multiplex cytokine analysis was undertaken using a high sensitivity human cytokine kit (EMD Millipore's MILLIPLEX MAP Human High Sensitivity Cytokine/Chemokine Panel, Massachusetts, USA). Multiplex analysis is a relatively new technology which is useful for high throughput analysis without the need for large sample volumes as is the case for ELISA. It is also relatively cost-effective and has been found to decrease experimental variability (268). The multiplex bead based kit measured levels of IL-1b, interleukin-2 (IL-2), IL-6, interleukin-7 (IL-7), interleukin-8 (IL-8), IL-10, interleukin-12 (IL-12), interferon-gamma (IFN-γ), granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α and NE. The cytokines were measured according to the manufacturer's instructions by laboratory personnel experienced in performing such analyses. For each sample, 50uL of serum was used. Standards and samples were assayed on a robotic liquid handling workstation (epMotion 5075, Eppendorf, Germany) and 96-well assay plates were washed with the Bio Plex II Pro wash station (Bio-Rad, California, USA) for magnetic beads and reported with Bio Plex Systems 100 software (Bio-Rad, California, USA). Intra-assay variability is quoted as 2-13%.

2.6.2.4 Nutritional analysis

Participants were required to fast overnight and arrive at the research clinic between 7:00 AM and 8:00 AM, where they first underwent assessment of REE followed by assessment of body composition and dietary intake. To mimic the resting state, other conditions adhered to included provision of transport and avoidance of intensive physical activity on the day before measurements.

2.6.2.4.1 Assessment of resting energy expenditure

Resting energy expenditure was measured by a trained technician blind to allocation using the MedGem (Analyser 3.0.1, 2004, HealtheTech, Inc, CO), a handheld indirect calorimeter measuring oxygen consumption and assuming a constant respiratory quotient (RQ) value of 0.85. Before each test the MedGem performed an automatic calibration, a five-second time period during which the ultrasonic flow sensors were set (HealtheTech User guide for MedGem Analyzer 3.0.1). Measurements of REE using the hand-held MedGem device were taken after a 10 minute rest and performed in a quiet environment with the subject resting comfortably in a seated position. The measurement required participants to create a leak-free seal between their mouth and a disposable scuba-type mouthpiece or mask covering both the nose and mouth. Measurements ceased when a 5-minute steady-state period was achieved or after 10 minutes, whichever occurred first. Once steady state breathing was achieved by participants (between 5-10 minutes) the Analyzer displayed REE (kcal/day) and VO_2 (ml/minute) on an LCD screen and measurements were documented. The analyzer calculated REE from oxygen consumption VO_2, a constant RQ value of 0.85 and grams of urinary nitrogen calculated from the average energy and protein intake of the United States population using the following modified Weir equation (269).

REE (kcal/day) = (3.941 x VO_2) + (0.85 x 1.106 x VO_2) – (2.17 x grams urinary nitrogen),

VO_2 is measured in L/day, grams of urinary nitrogen are calculated by [(kcal/day x 1.16) / 4] / 6.25.

Resting Energy Expenditure in kcal/day was converted to kilojoules by multiplying by a factor of 4.2 and then adjusted by kilograms body weight.

2.6.2.4.2 Assessment of body composition

Dual-energy X-ray absorptiometry (DEXA) is a non-invasive, safe, accurate and reliable method of body composition assessment in research and clinical practice (270-272). Whilst first developed for the assessment of bone mineral density, DEXA also provides an assessment of total and regional body fat and fat free mass (FFM) (270-273).

Whole body and regional body composition were estimated using DEXA (Lunar Prodigy, GE Healthcare, UK) with the automated reporting GE EnCORE bone densitometry software (version 10.51.006). The system software provided estimates of FFM, lean soft tissue, fat mass (FM) and bone mineral density for total body and body segments including both arms, both legs and the trunk. The DEXA technique uses an x-ray generator which emits alternating pulsed radiation of two photon energy peaks, 38KeV and 70KeV in a fan-beam mode. As photons transverse through human tissue, physical interactions occur, reducing the intensity of the beam, a phenomenon known as attenuation. This method allows for the differentiation between bone mineral and soft tissue densities because of the differences in absorption of the two photon energies.

Quality-assurance and quality-control measures for the DEXA were performed three times per week throughout the duration of the study and prior to all participant scans using a body composition phantom block containing a known bone mineral density and bone mineral content value. A tolerance for the densitometer was established from the mean of the

phantom bone mineral density by monitoring the densitometers performance, using ± 1.5% as the acceptable tolerable limit.

All DEXA scans were performed by a licensed technician who was blind to allocation. Participants were dressed in light clothing and requested to remove all metal containing accessories. Participants were positioned in a supine position on the DEXA table top within the defined boundaries with their feet in a neutral position and hands flat by their sides. Detection of internal metal devices, such as artificial joints, allowed for exclusion from calculations prior to analysis. Appendicular lean soft tissue mass from DEXA was used to determine skeletal muscle mass (SMM) according to an established equation (274):

Total-body SMM = (1.13 x Appendicular lean soft tissue) – (0.02 x age) + (0.61 x sex) + 0.97 (where 0=female and 1=male)

2.6.2.4.3 Assessment of dietary intake

Participants self-administered the Dietary Questionnaire for Epidemiology Studies Version 2 (DQES v2)(179) pre and post intervention. The DQES v2 was originally developed from a food frequency questionnaire utilised in the early 1980s to assess dietary intake of participants in the Melbourne Collaborative Cohort Study. Validation of the DQES v2 has been completed with comparisons made against seven-day weighed food records in a group of 63 women aged 16-48 years with findings suggesting correlation coefficients for nutrient intakes comparable with those reported for other commonly administered food frequency questionnaires. Further validation of the DQES v2 was performed by Xinying et al (275), this study also demonstrating that the DQES v2 can capture similar nutrient intake data as weighed food records and may be used for estimation of dietary intakes over a relatively short time in clinical intervention trials (275). The DQES v2 has also demonstrated acceptable levels of repeatability (276).

The DQES v2 includes:

74 food items with 10 frequency response options ranging from ‘Never’ to ‘3 or more times per day’;

3 photographs of scaled portions for four foods (used to calibrate portion size);

Questions on the overall frequency of consumption of selected fruits and vegetables (used to calibrate for overestimation of these foods); and

Questions on consumption of other selected foods that do not fit easily into the frequency format.

The 74 food items included in DQES v2 are grouped into four categories: 1) cereal foods, sweets and snacks; 2) dairy products, meats and fish; 3) fruits and 4) vegetables. A separate set of questions covers consumption of alcoholic beverages. The food composition data used to calculate nutrient intake are derived from *NUTTAB95* (277), with supplementation of other data where necessary (278-281).

Nutrients available for analysis from the DQES v2 included the following: water, energy, fat (total), sugars, starch and dextrin, fibre, cholesterol, sodium, potassium , calcium, phosphorus, magnesium, iron, zinc, retinol equivalent, retinol, thiamine, beta-carotene equivalent, riboflavin, niacin, niacin equivalent, vitamin C, alcohol, total saturated fatty acids, total monounsaturated fatty acids, total polyunsaturated acids, individual fatty acids, carotenoids, glycaemic index and glycaemic load.

The Australian and New Zealand Acceptable Macronutrient Distribution Range and Suggested Dietary Targets (SDT’s) for lowering chronic disease risk were selected for the dietary intake of participants to be compared against.

2.6.2.5 Skeletal muscle protein expression (calpain/calpastatin)

2.6.2.5.1 Muscle biopsy technique

The needle biopsy method, which Bergstrom invented 40 years ago (282), has now come to be widely used in studies of human skeletal muscle. The procedure has been proven safe to obtain muscle tissue with few complications (283) and with the introduction of this method, studies regarding human metabolic adaptation of the skeletal muscle to exercise and the adaptation mechanism of the skeletal muscle to exercise training have advanced dramatically (284).

Under ultrasound guidance (SonoSite M-Turbo, SonoSite, Inc, Bothel, USA), vastus medialis and medial gastrocnemius muscle of the symptomatic leg were imaged to ascertain the required depth and angle of biopsy needle in order to maximise yield and identify a location suitable to avoid visible vessels, thus minimising the risk of post-procedural haematoma and associated pain. In cases where both legs were symptomatic, the leg with lowest resting ABPI was chosen. Once an entry point was established, a generous amount of topical local anaesthetic cream was applied (Emla 5% cream, AstraZeneca, North Ryde, NSW, Australia; Lignocaine 25mg/g, Prilocaine 25mg/g) to anaesthetise the skin and facilitate reduced pain for the remainder of the procedure. Using aseptic technique, local anaesthetic (2% lignocaine, 100mg in 5ml; Pfizer) was then infiltrated into the sub-cutaneous and muscle tissue underlying the entry point at both locations on the leg. Given the short acting nature of such an anaesthetic agent, adequate analgesia was achieved within 2-3 minutes, at which point a 15 gauge x 7.8cm disposable co-axial biopsy needle with depth stop (Bard TruGuide, Bard Peripheral Vascular, Tempe, Arizona, USA) was inserted percutaneously at the appropriate angle to the required depth. The trochar was removed and replaced by a 16 gauge x 10cm disposable core biopsy instrument with a penetration depth of 22mm and 1.9cm length of sample notch (Bard MaxCore, Bard Peripheral Vascular, Tempe, Arizona, USA). This was

deployed on five occasions in both vastus medialis and medial gastrocnemius muscles allowing collection of skeletal muscle. Muscle samples were immediately rinsed of residual blood in isotonic saline, before being snap frozen in liquid nitrogen and transported for storage in a -80° freezer. Upon completion of the procedure, firm, direct pressure was applied for several minutes to each biopsy site, before an ice pack and pressure bandaging was applied in an attempt to minimise pain, swelling and haematoma. This was left in place for four hours after the completion of the procedure. In the event of any complication, patients were asked to contact the candidate on the number listed in the patient information sheet.

2.6.2.5.2 Preparation of muscle samples and extraction of protein

Skeletal muscle samples obtained were selectively assessed for protein activity of proteins from the calpain family, specifically, m-calpain and calpastatin. This first required the extraction of protein from muscle samples.

Muscle was initially weighed to identify wet weight before being again washed in isotonic solution to remove excess blood (a source of high levels of plasma protein which may compromise accuracy of analysis) and pulverised with mortar and pestle in liquid nitrogen.

In order to extract protein from the muscle, a homogenisation buffer (HB) was mixed to the now pulverised muscle. Based on previous work to assay calpain/calpastatin activity in skeletal muscle of rodents (174), the HB contained a basic buffer solution, protease inhibitors and dithiotreitol (DTT) to reduce the disulphide bonds of proteins. The constituents of the HB are listed below. Most were prepared firstly as stock solution for storage purposes before being mixed together in smaller volumes to form the HB when required.

- A 100ml stock solution of buffer was made up consisting of distilled water, Tris, Ethylenediaminetetracetic acid (EDTA) and hydrochloric acid (HCl). Tris is a common component of buffer solutions with a pKa of 8.07 at 25℃. The required

concentration for the stock solution was 4.84g/L (40mM). Therefore 484mg was required per 100mls of buffer. Ethylenediaminetetracetic acid is a chelating agent with ability to sequester metal ions such as calcium. Such an action was critical to the accuracy of an assay to assess the activity of a calcium-dependent protein system. Required concentration for the stock solution was 10mM and pre-mixed stock solution available was 500mM. Therefore in a 100ml solution, 2mls of 500mM EDTA was required. Distilled water was then added to bring the volume to 100mls. Hydrochloric acid was then slowly titrated into the buffer with the guidance of a pH meter and spinning chip to achieve a final pH of 8.0.

- E-64 is an irreversible and highly selective inhibitor of cysteine proteases. Although calpains are cysteine proteases, they are not inhibited by E-64 in the presence of EDTA, except when Ca^{2+} is added to the solution. This is proposed to be due to the conformation of their active site in the absence of Ca^{2+}. Its inclusion in the HB was an effective method of inhibiting cysteine proteases while purifying the calpains. Stock solution was made in 50/50 ethanol/water at a concentration of 10mg/ml. E-64 (5mg) was obtained from Sigma-Aldrich Australia and added to 500uL of 50/50 ethanol water solution to make 10mg/ml. This was equivalent to a molarity of 28mM (molar mass = 357.41). For storage purposes, 100 x 5uL aliquots of 28mM E-64 were prepared.

- Stock solution of trypsin inhibitor was prepared using distilled water and stored at -80°C in aliquots at a concentration of 1mg/ml. Trypsin inhibitor was purchased in powder form from Sigma-Aldrich Australia.

- The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was prepared with 2-propanol to achieve a stock solution at 100mM (required 174.2mg PMSF to be

added to 10mls of 2-propanalol). Due to its sensitivity to light, it was then wrapped in alfoil and stored at 4°C.

- Dithiotreitol was used to reduce the disulphide bonds of proteins and was available in powder form.

The volume of HB required was based on the assumption that 200mg of muscle gives approximately 10% wet weight of protein = 20mg protein. Therefore to achieve a concentration of 10mg/ml muscle extract, 2mls of HB was required for each muscle sample. Five muscle samples were able to be processed/analysed on any given day. Therefore on each day of testing 10mls of HB was prepared. This required appropriate dilutions of stock solutions to be performed, such that the HB consisted of 20mM Tris-HCl (pH 8.0), 5mM EDTA, 0.1% DTT, 100mg/L trypsin inhibitor, 2.8uM E-64 and 2mM PMSF.

Each 10mls of HB therefore contained 5mls of Tris-HCl-EDTA buffer, 1ml of trypsin inhibitor stock solution, 10mg DTT powder, 1ml of E-64 (after stock aliquots were diluted 1:1000 by the addition of 5mls distilled water to a 5uL aliquot), 100uL PMSF and 2.9mls distilled water.

The HB (2mls/muscle sample) was then mixed with pulverised muscle before samples were subjected to high speed centrifugation (20,000g at 4°C for 30 minutes). Each sample was balanced to within 1mg inside the centrifuge.

The supernatant from each sample was then collected into eppendorf tubes and stored on ice while preparations were made to calculate the protein concentration of samples using the EZQ protein quantification assay.

Using a 2.0mg/ml stock solution of ovalbumin, dilutions were made to achieve concentrations of 2.0, 1.0, 0.5, 0.2, 0.1, 0.05 and 0.02mg/ml. These facilitated construction of

a standard curve. All muscle samples were diluted by 1:4 using distilled water to ensure concentrations did not exceed the standard curve. A sheet of assay paper was placed onto a microplate with a backing plate over it and 1uL of each standard, sample and blank were pipetted in triplicate onto the assay paper. Once dry, the assay paper was placed in a tray and 40mls of methanol added, before the tray was stirred at 50rpm for 5 minutes. The methanol was then poured off and paper was dried on low heat before 35mls of EZQ protein quantification reagent was added and stirred for a further 30 minutes. After the reagent was poured off, 40mls of 10% methanol and 7% acetic acid in distilled water was added and stirred for 2 minutes. This step was repeated for a total of three rinses. The wet assay paper was then placed face down in a Typhoon FLA 7000 Scanner (GE Healthcare Life Sciences, Cleveland, Ohio, USA) for analysis. Fluorescence data was obtained and transferred into a Microsoft Excel spreadsheet enabling standard curves to be constructed and subsequently the protein concentration of each sample to be determined.

2.6.2.5.3 Determination of calpain activity

Once protein was extracted from muscle samples and its concentration in the supernatant determined, calpain activity could be determined using 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid labelled casein (BODIPY-FL-casein) as the calpain substrate. This validated method releases highly fluorescent labelled peptides that are released following cleavage by activated proteases (ie calpains), resulting in an increase in fluorescence which can be measured with a microplate reader.

For each sample, skeletal muscle extract (containing 50ug protein) was added to a microtitre plate well (in duplicate) and dilution buffer (containing 20mM Tris-HCl (pH 7.5), 1mM EDTA, 100mM potassium chloride (KCl) and 0.1% DTT) was added to bring the total volume to 100uL. Dilution buffer was prepared as a 200ml stock solution in which 484mg Tris was added to 400uL of 500mM EDTA stock solution before distilled water was added to

bring the volume to 200mls. 1.49g KCl was then added to the solution and as described for HB, HCl was then titrated drop by drop to achieve a pH of 7.5. Dithiotreitol was added to the solution on the day of testing such that 1mg/ml was required to achieve a concentration of 0.1%. 100uL of reaction buffer (containing 16ug/ml BODIPY-FL-casein, 20mM Tris-HCl (pH 7.5), 1mM EDTA, 10mM Calcium chloride (CaCl2), 100mM KCl and 0.1% DTT) was then added to each well to initiate the reaction. Preparation of the reaction buffer was the same as that described for the dilution buffer with the addition of calcium chloride and BODIPY-FL-casein. Based on a molecular weight of 110.98gm, to achieve a concentration of 10mM 220mg of calcium chloride was added to the buffer. BODIPY-FL-casein was prepared according to the manufacturer's instructions (LifeTechnologies Australia, Mulgrave Victoria). Importantly, although E-64 was still present in the muscle extract, it was diluted to an extent that it was not inhibitory to the calpain system despite the addition of calcium.

On the same plate and for each sample, this process was repeated except that calcium chloride was omitted and 100mM EDTA was added to the reaction buffer. Plates were incubated at 25°C for 60 minutes, before the reaction was stopped by adding 25uL 100mM EDTA to each well. Fluorescence was read at 485nm excitation and 530nm emission wavelengths. Calpain activity was expressed as fluorogenic units and was calculated as the difference between activity measured in the presence and absence of calcium in the reaction buffer, based on the rate at which calpain degraded BODIPY-FL-casein to release fluorogenic peptides.

2.6.2.5.4 Determination of calpastatin activity

To assess calpastatin activity, aliquots of homogenised muscle extract were heated to 100°C for five minutes in order to denature calpain proteins (calpastatin proteins are stable at this heat). At this temperature, denatured proteins solidify, therefore after heating, high speed centrifugation was performed (*See section 2.6.2.5.2* Preparation of muscle samples and

extraction of protein) and the supernatant collected and allowed to cool to room temperature. The volume of supernatant containing 50ug protein (volume determined by EZQ assay before heating) was added to microtitre plate wells and 800ng of purified u-calpain (Calbiochem, California USA) ie 12.4uL was also added. Dilution buffer (*See section 2.6.2.5.3* Determination of calpain activity) made the volume in each well up to 100uL. The reaction was started with the addition of 100uL reaction buffer (S*ee section 2.6.2.5.3* Determination of calpain activity). On the same plate and for each sample, this process was repeated except that muscle extract containing calpastatin was omitted and an equivalent additional volume of dilution buffer added. Plates were incubated at 25°C for 60 minutes, before the reaction was stopped by adding 25uL 100mM EDTA to each well. Fluorescence was read at 485nm excitation and 530nm emission wavelengths. Each test was performed in duplicate. Calpastatin activity was calculated as the %inhibition of calpain activity in those samples in which muscle extract was present compared to those in which muscle extract was omitted.

2.6.2.5.5 Validation of technique

Before the above assays were performed on human skeletal muscle samples, methods were first trialled on skeletal muscle harvested from mice and the linearity of the assay was assessed. Mice used were all female Balb/c inbred albino straw mice, aged between 17-18 weeks. Anterior and posterior thigh muscles were harvested opportunistically just prior to the sacrifice of these animals being used as part of another experiment which had ethics approval from the Southern Adelaide Health Service Animal Welfare Committee. Before the procedure was performed, the mice were anaesthetised using 75mg/kg ketamine and 0.3mg/kg medetomidine mixed in the same syringe. Sacrifice using cervical dislocation followed. All muscle samples were snap frozen in liquid nitrogen and stored at -80°C.

2.6.3 Quality of Life (QoL) assessment

The Australian Vascular Quality of Life Index (AUSVIQUOL) is a validated tool for the assessment of QoL of patients with PAD in the clinical setting (285). It consists of 10 questions addressing general health perceptions, functional mobility and pain and psychosocial aspects of health. For the purpose of the study presented in this thesis, each question had five possible responses listed in order such that the first response was most desirable and scored one point and the fifth response was least desirable and scored five points. Patients were asked to complete this questionnaire independently and a score out of 50 was recorded. Importantly, a lower value represents an improvement in QoL. A copy of the questionnaire is provided in *Appendix 2*.

2.7 Statistical Analyses

The primary analysis was undertaken on an ITT basis with those lost to follow-up at 12 weeks having their baseline measure carried forward. For per-protocol analysis, all participants achieving less than 80% adherence to the allocated SET and those that were lost to follow-up were excluded from the analysis. Statistical Package for Social Sciences version 19 (SPSS Inc, Chicago IL, USA) was used to perform statistical analyses. Continuous data are reported as mean (SD) or median (IQR) according to normality and categorical data as n (%). Differences within groups for continuous data were tested using paired samples t-test or Wilcoxon Signed Rank test and change from baseline to 12 weeks between groups using independent samples t-test or Mann-Whitney U test. Differences between groups for categorical data were tested using X^2. Relationships between variables were assessed using Pearsons or Spearmans correlation coefficient according to normality. Statistical significance of $P<0.05$ was assumed.

CHAPTER 3: PARTICIPANT RECRUITMENT, RETENTION AND ADHERENCE AND THE IMPACT OF THE INTERVENTION ON QUALITY OF LIFE AND THE PRIMARY OUTCOME OF PAIN FREE WALKING DISTANCE

3.1 Introduction

Interpretation of RCT findings requires attention to external and internal validity, specifically representativeness of sample, allocation bias and protocol adherence. Furthermore, the effect size of the intervention on the a priori primary outcomes will undoubtedly impact on the uptake of the findings in clinical practice.

For the purpose of this RCT the primary outcome is PFWD. Treatment priorities for patients with intermittent claudication include: (a) risk factor modification to prevent disease progression and future cardiovascular events, and (b) symptomatic and functional improvement and psychosocial health as determined by pain-free and maximal walking performance and QoL assessment. Treadmill based supervised exercise training is currently regarded as the gold-standard treatment to provide measurable improvement in walking performance (7), however, recent evidence has emerged suggesting that other exercise modalities, including resistance training, may provide benefits that are comparable to treadmill training (236). This raises the possibility of an additive benefit of exercise training regimens consisting of a combination of exercise modalities. There are currently no studies that have assessed the impact of SET with both treadmill training and lower limb resistance exercise compared with treadmill training alone on PFWD and QoL.

3.2 Aim

The aim of this chapter is to report on participant recruitment, retention and adherence and the impact of the intervention as it relates to RQ1: "Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant improvement in **PFWD** and **QoL** compared to an exercise regimen consisting of treadmill training alone in patients with IC?"

3.3 Participants

3.3.1 Recruitment

Between February 2011 and July 2012, 111 new patients were referred to the claudication clinic. Sixty-five patients were deemed to be unsuitable for the program, many of whom were found to have an alternative diagnosis accounting for their symptoms, including venous pathology (n=3), lumbo-sacral spinal pathology (n=9) eg canal stenosis or other nerve root impingement and other musculoskeletal pathology (n=6) eg osteoarthritis or muscular strains. The major reason for patients being excluded from the SET was the presence of proximal (ie aorto-iliac) disease (n=28). Other reasons included disease severity (n=8) ie with CLI; underlying cardio-respiratory co-morbidities (n=8); impaired mobility (n=1); recent surgery (n=1) and complete resolution of symptoms (n=1).

This resulted in 46 patients being considered suitable for enrolment into the SET, of whom 35 patients agreed to participate and 11 patients declined. The reasons given for declining to participate were: too much commitment (n=4); concurrent health issues that took precedence over the PAD (n=4) e.g. pending surgery, multiple other medical appointments; a preference for self-directed home-based exercise (n=2) and work commitments (n=1).

3.3.1.3 Demographics

The study population consisted of 35 participants, of which 18 (12M, 6F) were randomised to treadmill only training (Group A) and 17 (13M, 4F) to a combination of treadmill training and lower limb resistance exercises (Group B). There were no statistically significant differences between demographics in Group A and Group B (*see* Table 2*).* As expected, the majority of patients had a medical history that included hypertension, dyslipidaemia and cigarette smoking, while many also had a diagnosis of diabetes mellitus and/or ischaemic heart disease.

All patients were taking anti-platelet and lipid lowering therapy prior to presentation to the claudication clinic or were prescribed such therapy after diagnosis of PAD at least 2 weeks before commencement of the SET. No patients had contra-indications to these medications. In addition, the majority of patients (n=14 in Group A and n=15 in Group B) were also taking anti-hypertensive agents.

3.3.1.4 Disease Severity

All 35 participants recruited had a diagnosis of intermittent claudication defined by Rutherford's classification of PAD as category 1-3. Lowest resting ABPI and baseline PFWD were not significantly different between groups (*see Table 2*).

3.3.1.5 Retention of participants

Figure 3 illustrates the flow of participants from enrolment into the study to follow-up after completion of the allocated 12 week SET. While five and three participants in Group A and Group B respectively did not complete the study, three and two returned for a follow-up assessment and hence contributed data for ITT purposes and two and one did not return for a follow-up assessment and hence had their baseline measure carried forward to enable ITT analysis. Of the participants who did not complete the

study, six participants withdrew due to the onset of medical conditions including angina, diabetic foot infection, shingles, transient ischaemic attack, urosepsis and disc prolapse. One participant withdrew on the premise that he "did not see any value in completing the program", while one participant failed to attend the number of sessions (80%) required for satisfactory completion of the program.

3.3.2 Participant Adherence to Training

Mean adherence to the SET was 84.5% (SD 27.3) and 82.8% (SD 21.0) for participants in Group A and Group B respectively (P=0.84). Reasons cited for non-attendance commonly included illness, holidays or work-related commitments.

Table 2: Baseline characteristics between participants in the treadmill only supervised exercise training and the treadmill and resistance exercise supervised exercise training. Data presented as n (%) unless otherwise stated.

Demographics and medical history	**Group A (n=18)**	**Group B (n=17)**	**P-*value***
Age (years) – Mean (SD)	73.4 (9.1)	69.4 (9.6)	0.22
Male:Female ratio	12:6	13:4	0.52
Current/ex-Smoker	13(72)	13 (76)	0.77
Body Mass Index, kg/m^2 – Mean (SD)	27.0 (4.3)	29.0 (5.6)	0.39
Ischaemic Heart Disease	6 (33)	8 (47)	0.41
Dyslipidaemia	16 (89)	16 (94)	0.58
Hypertension	14 (78)	15 (88)	0.41
Diabetes Mellitus	6 (33)	10 (59)	0.13
Resting Ankle Brachial Pressure Index – Mean (SD)	0.71 (0.23)	0.72 (0.15)	0.97
Pain Free Walking Distance, metres – Mean (SD)	159.7 (83.9)	180.6 (90.5)	0.48

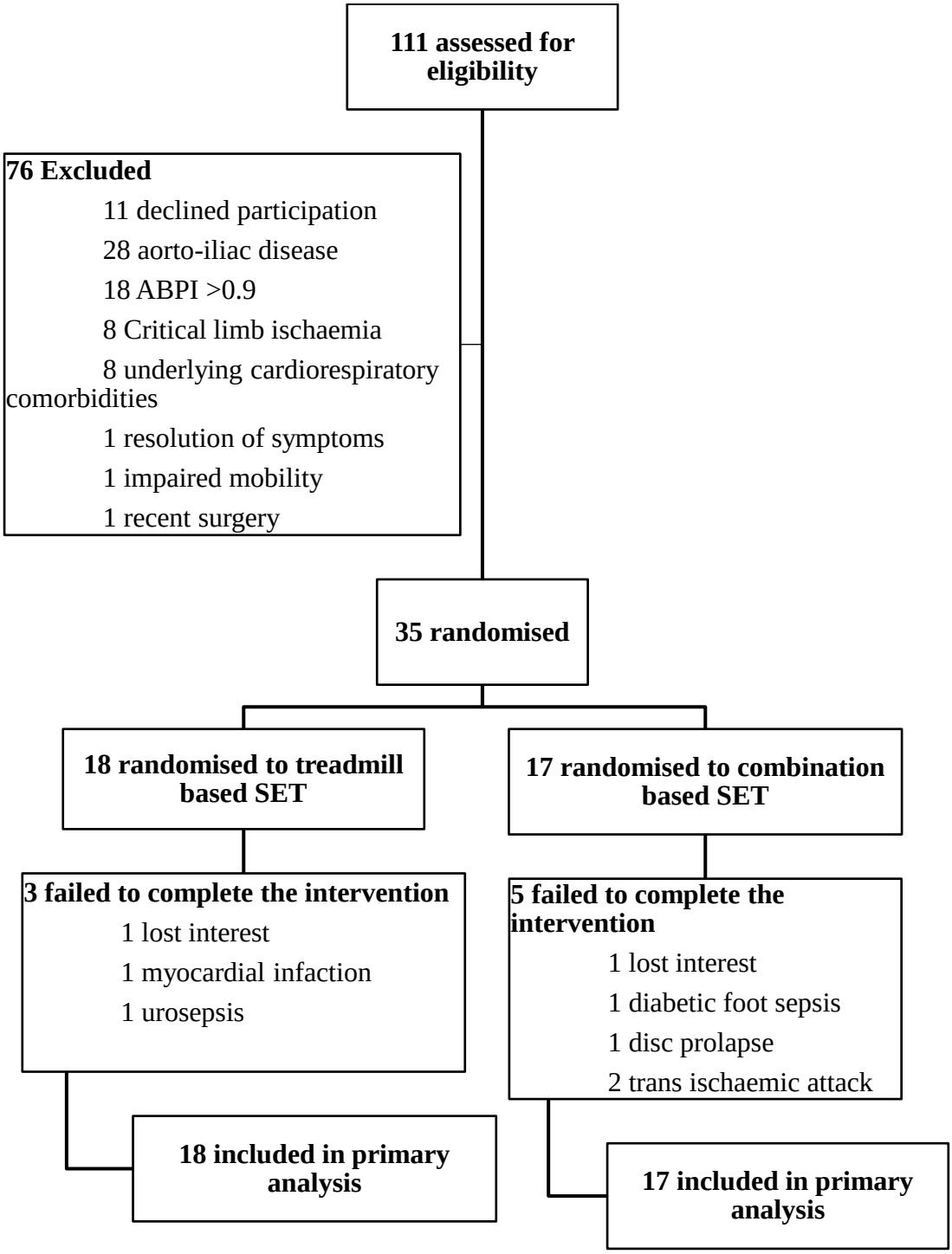

Figure 3: CONSORT diagram illustrating flow of participants through the trial.

3.3.4 Exercise Intensity

Table 3 highlights that there was no significant difference in exercise intensity between Group A and Group B at the commencement of the SET and the same was true at the end of the SET. In addition, no within group difference was observed. Translated into perception of effort, these scores at both commencement and on completion of SET represent "somewhat hard" to "hard" exercise.

3.3.5 Walking Performance

Table 3 highlights the change in the outcome measures for walking performance from baseline to 12 weeks according to allocation, for both ITT and per protocol analyses. There were no statistically significant differences observed between groups at baseline for any of the outcome measures.

Pain free walking distance increased significantly between baseline and 12 weeks for Group A, both on ITT and per-protocol analysis. A small increase was achieved between baseline and 12 weeks for Group B however this did not achieve statistical significance on either ITT or per-protocol analysis and there was no between group difference for the increase observed in PFWD.

Similar to the findings for PFWD, on both ITT and per-protocol analyses, 6MWD improved in both groups between baseline and 12 weeks however this only achieved statistical significance for those participants allocated to Group A. There were no between group differences identified for either ITT or per protocol analyses.

Table 3: Intention to treat and per protocol analysis demonstrating between and within group differences for those participating in either the 12 week treadmill only supervised exercise program or the 12 week treadmill and resistance training supervised exercise program: walking performance, quality of life and rating of perceived exertion

Outcome	Treadmill only (Group A)				Treadmill and Resistance Training (Group B)				Between groups P-*value*
	Baseline	12 Weeks	Change	P-*value*	Baseline	12 Weeks	Change	P-*value*	
Intention to treat									
PFWD, metres	160 (84)	204 (97)	44 (80)	.03	181 (90)	188 (109)	7 (135)	.82	.42
6MWD, metres	354 (99)	386 (85)	34 (47)	.01	368 (69)	419 (168)	51 (165)	.21	.67
QoL	20.4 (3.3)	18.7 (4.2)	-1.6 (2.4)	.01	22.3 (4.0)	19.0 (4.6)	-3.3 (3.9)	.01	.18
Per protocol									
PFWD, metres	170 (82)	221 (97)	50 (78)	.03	170 (90)	188 (127)	18 (147)	.67	.59
6MWD, metres	371 (94)	409 (70)	40 (49)	.01	358 (68)	439 (188)	81 (189)	.16	.47
QoL	19.5 (2.2)	17.6 (2.9)	-1.9 (2.5)	.01	21.9 (3.6)	19.1 (5.0)	-2.8 (3.7)	.02	.45
Borg RPE score	14.1 (1.9)	14.9 (2.5)	0.8 (2.1)	.83	14.3 (2.2)	13.5 (3.1)	-0.8 (2.7)	.23	.31

Table 4: Individual level data for the walking performance outcomes.

Study ID Number	Allocation	Baseline PFWD	Post SEP PFWD	Change in PFWD	Baseline 6MWD	Post SEP 6MWD	Change in 6MWD
2	Group A	120	160	40	245	320	75
4	Group A	200	160	-40	285	295	10
6	Group A	120	80	-40	320	370	50
7	Group A	200	240	40	400	457	57
8	Group A	160	240	80	501	492	-9
9	Group A	400	473	73	455	473	18
12	Group A	240	240	0	355	380	45
15	Group A	35	160	125	188	220	32
18	Group A	200	120	-80	360	338	-22
21	Group A	120	120	0	560	520	-40
22	Group A	80	240	160	285	395	110
23	Group A	80	200	120	285	400	115
24	Group A	80	80	0	260	260	0
25	Group A	160	200	40	420	500	80
29	Group A	120	160	40	240	320	80
30	Group A	200	320	120	420	435	15
31	Group A	240	160	-80	403	360	-43
34	Group A	120	320	200	384	415	31
1	Group B	240	120	-120	290	320	30
3	Group B	120	160	40	350	273	-77
5	Group B	280	280	0	380	380	0
10	Group B	180	180	0	390	390	0
11	Group B	80	160	80	430	465	35
13	Group B	80	520	440	315	978	663
14	Group B	120	120	0	340	350	10

16	Group B	120	120	0	320	353	33
17	Group B	160	80	-80	305	325	20
19	Group B	330	120	-210	330	289	-41
20	Group B	160	200	40	300	430	130
26	Group B	80	120	40	317	280	-37
27	Group B	280	180	0	440	390	-50
28	Group B	240	160	-80	335	355	20
32	Group B	120	120	0	400	435	35
33	Group B	120	200	80	517	538	21
35	Group B	360	360	0	500	588	88

Table 4 displays the walking performance data for each participant in the RCT. Of note, only 10 (55%) participants in Group A improved their PFWD while 6 (35%) improved their PFWD in Group B.

3.3.6 Quality of Life (QoL) Assessment

Table 3 highlights the change in the outcome measure for QoL from baseline to 12 weeks according to allocation, for both ITT and per protocol analyses. There were no statistically significant differences observed between groups at baseline.

Significant improvements in QoL score were reported in Group A and Group B for both ITT and per-protocol analyses. The improvement noted between groups was comparable with no statistically significant difference observed.

3.3.7 Summary of findings

In summary, this RCT has demonstrated that in patients with IC, 12 weeks of SET consisting of treadmill training alone can produce improvements in walking performance but this is not superior to the non-significant change in walking performance observed in patients undertaking a combination of treadmill training and lower-limb resistance exercises. Exercise in both groups lead to improvements in QoL and acceptable levels of adherence to the SET were achieved.

3.4 Discussion

Consensus guidelines recommend that treadmill based supervised exercise training should be made available as the first line of treatment for all adults with IC on the basis of extensive high quality evidence demonstrating improvement in both pain free and maximum walking performance following SET of sufficient intensity to induce claudication pain (7).

Similar to treadmill based training alone, resistance training alone has been shown to improve walking performance in patients with IC (236). Overall improvements in cardiovascular conditioning and a gain in SMM leading to greater functional capacity are likely responsible for this. A comparative group consisting of lower-limb resistance training combined with treadmill based training was therefore chosen for the study presented in this thesis as it was anticipated that the additive effect of mechanisms responsible for improvement in both treadmill and resistance training may lead to greater improvements in walking performance.

Although a significant improvement in walking performance was observed within the treadmill only exercise group, this was not superior to the non-significant change observed in participants undertaking a combination of treadmill and lower-limb resistance training. The lack of an observed difference between the groups in our study was in contrast to our expectation that the additive effect of treadmill training and resistance exercise would lead to superior improvements in walking performance when compared with treadmill training alone. There are several reasons why this hypothesis may not have held true.

3.4.1 Duration of relative ischaemia

Participants in the treadmill-only group (Group A) were expected to walk to MWD before resting, while those undertaking combination training (Group B) walked only to the onset of pain. Although this induced a relative ischaemia in both groups, the duration of ischaemia was greater in Group A, potentially maximising the pro-angiogenic stimulus and facilitating a greater opportunity for skeletal muscle metabolic adaptation to occur, both of which have been proposed as key mechanisms to explain the clinical improvements in response to treadmill based SET (122, 164). Although this was understood at the time of protocol development, a strategic

decision was made to limit the walking undertaken by participants in Group B to PFWD only, with the view that this would potentially limit IRI and the resultant pro-inflammatory response and detrimental effect on endothelial function, measured as secondary outcomes for this trial (*See Chapter 4 and 5).* At the same time, it was hypothesised that the additive effect of resistance training would at the very least compensate for a reduced ischaemic stimulus. Such a decision was supported by work from Mika et al (127) who demonstrated that three months of pain-free treadmill walking could significantly improve walking performance compared with a non-exercising control group, without evoking a systemic inflammatory response. This suggests that mechanisms exist to improve walking performance in patients with IC independent of those associated with the ischaemic response that are currently recognised. Support for such a concept was not provided by the findings of this thesis.

3.4.2 Volume of exposure to exercise

The American College of Sports Medicine (ACSM) guidelines (97) suggest that it is the volume of energy that is expended rather than the duration of exercise that is an important determinant of exercise response in chronic disease patients. To highlight this, relevant to aerobic activity (ie treadmill training) in the general population, an energy expenditure of approximately 1000 kilocalories per week (kcal/wk) is associated with lower rates of CVD and premature mortality (97). There are currently no guidelines in existence specifying a threshold value of exercise based energy expenditure associated with adaptive responses and improved walking performance in patients with IC.

An accurate assessment of exercise induced energy expenditure requires specialised equipment to measure oxygen consumption allowing conversion to kilocalories. More commonly, a measure of exercise intensity is used as a marker of energy expenditure

relative to an individual's maximum level of function. This is recommended for older patients and those with chronic disease. Intensity itself has been proposed as an important determinant of the physiological response to exercise training. Recent pilot work from Parmenter et al (286) has demonstrated that high intensity resistance training produces a superior improvement in walking performance compared with low intensity training of the same duration. To further investigate this concept, Gardner et al (130) controlled for energy expenditure by ensuring that those engaging in high intensity exercise were active for shorter periods. No difference was observed between the high and low intensity groups, suggesting that the improvements in walking performance associated with high intensity exercise observed in Parmenter et al (286) are likely related to an increase in energy expenditure associated with high intensity exercise conducted over the same duration as low intensity exercise.

The Borg Rating of Perceived Exertion Scale (264) was utilised in this RCT to assess intensity and the results suggest that exercise intensity and subsequently energy expenditure (as participants in each group exercised for the same duration) was similar in both groups at the beginning and the end of the SET. This energy expenditure was however, shared across two exercise modalities in Group B and it is possible then that in a quest to successfully combine two exercise modalities, participants in Group B were simply not exposed to a sufficient volume of either treadmill or resistance training to enable an adaptive response to either modality to occur. A similar conclusion was reached by Treat-Jacobsen et al (121), who also failed to demonstrate an additive effect of combining arm-cranking and treadmill training.

To overcome this and achieve the maximal additive benefit of combination training that was expected to be seen, consideration could be given to increasing the intensity

of exercise prescription while maintaining the same frequency and duration of session, or alternatively, additional sessions could be scheduled in which the intensity of exercise remains constant. Of course, any increase in frequency or duration would need to be replicated in both groups and care must be taken when subjecting patients with CVD to high intensity exercise.

Although it seems that exercise intensity, duration and therefore energy expenditure were well controlled for during supervised sessions, data were not collected on any additional self-prescribed exercise training undertaken by participants. While all participants were instructed not to engage in home-based exercise beyond their baseline level of physical activity, studies have utilised pedometers and accelerometers to demonstrate significant improvement in self-prescribed physical activity at the completion of SET (49, 117, 120). Whether or not this is simply motivation and confidence to walk further with pain or is actually reflective of improvements in walking performance derived from the SET remains to be seen. This does suggest that even throughout the duration of the SET, participants are likely to be undertaking additional external physical activity which may be influencing the total volume of energy expenditure throughout the SET and subsequently the outcome. In the future, this should be addressed by collecting these data using exercise diaries requiring participants to document daily physical activity, type, duration and intensity.

3.4.3 Variability of response to exercise

A striking feature of the results of this RCT is the huge variability in response to exercise, as is demonstrated by the standard deviations associated with the change in both PFWD and MWD. In fact, it is evident that the SET (both Group A and Group B) has had a detrimental impact on walking performance in several individuals.

Reasons for such variability in response are poorly understood, but are likely to be multifactorial and determined by the mechanism(s) of action underlying the potential beneficial effects of exercise in patients with IC. Genetic factors are likely to be involved in angiogenic or metabolic adaptations while psychological factors such as the belief in the beneficial effects of exercise and motivation to achieve are also likely to play a role (165). Such variability in response has also been observed in Treat-Jacobsen et al (121) and to a lesser extent in almost all studies. It does raise the question as to whether or not there exists a biodiversity in these patients response to exercise and indeed whether there is a potential biomarker to identify patients who will or will not respond to exercise. While the search for biomarkers to represent disease progression, prognosis and response to treatment is evolving across a host of pathologies, including PAD, there is currently a paucity of literature investigating the role of biomarkers in predicting response to SET in patients with IC. Circulating levels of the cytokine IL-6 and other inflammatory markers such as CRP have been proposed as potential markers for disease progression in PAD (151, 202) but their role in predicting response to exercise remains unclear.

Irrespective of this, it is important to recognise that biomarkers are not highly sensitive or specific and it is likely that response to exercise, just as with the atherosclerotic disease process itself, is complex and governed by a multitude of factors including genetics, anatomical distribution of disease and endothelial function.

The impact of such variability in response to SET may serve to mask the potential benefits that could be observed from exercise training, in particular the combination training arm of the study. The mean improvement in 6MWD in Group B is double that of Group A, while with respect to PFWD, the mean improvement in Group B is less than one-sixth that of Group A. Despite these quite obvious trends, the data were

unable to demonstrate statistical significance between groups and thus, conclusions regarding the relative superiority of one modality over the other are unable to be reached. It is likely that the marked variability and subsequent large standard deviation is a key reason for this lack of statistical significance.

Although recruitment exceeded the numbers required for sufficient statistical power on the basis of an *a priori* power calculation, the observed effect size was less and the standard deviations were greater than what had been used in this calculation. It is possible therefore that this study may have been underpowered and future studies should give consideration to an inflation of sample size. A post-hoc power calculation based on the findings of this work, including an observed drop-out rate of 20%, with alpha = 0.05 and beta = 0.80, demonstrates the need for a sample size of 82 participants per group.

3.4.4 Comparison of walking performance data with current literature

The findings of this RCT are unique in that there are no previous RCT's comparing the impact of treadmill based SET with a combination of treadmill training and lower limb resistance exercises.

While the improvement observed in PFWD in the treadmill only group (44m) is only half that reported by the Cochrane review (82m) (110), direct comparison of results from within groups is challenging due to the heterogeneity of protocols and methods of assessment demonstrated throughout the literature. In this RCT the 6MWT was selected for assessment of walking performance to avoid the potential learned effect associated with standardised treadmill based-protocols and with the view that a land-based test would best represent the actual functional capacity and therefore the meaningful improvements conferred by SET (122). Despite this, only five studies

have utilised the 6MWT as a method of assessment (113-115, 122, 130), of which, only McDermott et al (122) have employed 6MWT as the primary outcome assessment, while only Gardner et al (114) measured PFWD using the land-based 6MWT. There are no previous studies that have utilised the 6MWT to assess the impact of combined treadmill training and lower-limb resistance exercises on walking performance. In fact, only one study has previously assessed the impact of such a combination training program on walking performance. Using a graded-treadmill test, Hiatt et al demonstrated that MWT but not PFWT improved significantly, although this was not compared to treadmill-training alone (123). With respect to a combination based SET, the results of the RCT presented in this thesis failed to demonstrate a significant improvement in either PFWD or 6MWD.

With respect to treadmill only training, the observed improvement in PFWD (40m) is only half of that demonstrated by Gardner et al (114) who used a similar protocol but over a six month duration. While this could lead one to question the duration of an exercise intervention required to obtain optimal benefit in walking performance, the observed improvement in 6MWD of 40m is comparable to that of the other studies that have utilised the 6MWT and in fact greater than McDermott et al (122) and Gardner et al (130), whose interventions lasted for six months. Consensus guidelines recommend between three to six months of supervised exercise (7), however, it is likely that exercise intensity, energy expenditure, compliance and genetic factors of participants are all variables that are just as important as duration when considering outcomes.

3.4.5 Differences between markers of walking performance and the impact of assessment methods

It has been proposed that different mechanisms may exist to account for changes in PFWD and MWD (112, 121). The results of the RCT presented in this thesis demonstrate that several participants improved their 6MWD while their PFWD remained constant. This suggests that an improved pain tolerance may be responsible for improvements in 6MWD or maximal walking performance, independent of any bio-molecular adaptations that may account for improvement in PFWD. Support for this concept is provided by Wood et al (125) who demonstrated significant improvement in MWT but not PFWT after 6 weeks of treadmill based SET. In addition, in the current study, Group B demonstrated a mean improvement in PFWD of only 7m, compared to 51m for 6MWD, while mean improvement in Group A was of similar magnitude for both PFWD (44m) and 6MWD (34m). A similar trend was identified by Treat-Jacobsen et al (121) who used arm-ergometry and treadmill training alone or in combination to obtain results that led her to conclude that PFWD may be influenced more by systemic adaptations than MWD which may be dependent on a combination of local muscle and systemic adaptations.

Interestingly, the results of the RCT presented in this thesis also demonstrate that within the entire cohort, three patients improved their PFWD while their 6MWD worsened, and a further five patients improved their 6MWD at the expense of deterioration in PFWD. Of course, in a test where time is constant, the distance walked is influenced only by speed and it is possible that those patients who improved only their PFWD may have slowed down to ease the metabolic requirements of the muscles. The opposite may have occurred for those who only improved their 6MWD. These findings are likely to represent an inherent flaw in the 6MWT rather than an

actual difference in mechanism underlying PFWD and MWD. On this basis, it could be argued that the time of such a test should not be constant and that for the purpose of assessing walking ability of patients with IC, a non time-limited land based test following the same or similar protocols as the 6MWT should be implemented. On the contrary, although this would provide information on maximal walking performance, evidence would suggest that 95% of walking activity in both healthy individuals and those with IC occurs in short bursts and therefore 6 minutes of constant walking is rarely exceeded (287) making MWD a somewhat unimportant measure that is not applicable to normal daily function.

3.4.6 Adherence to the SET

Despite the established benefits of exercise, modern lifestyle factors mean that a large proportion of the population fail to participate in the recommended levels of physical activity (97). Furthermore, exercise programs for chronic disease patients seem to be hindered by poor compliance often due to underlying co-morbidities (288). In contrast to this, the results of the RCT presented in this thesis have demonstrated a high level of compliance (>80%) to an exercise intervention, while the drop-out rate (20%) was also reasonable and only suffered from the fact that acute medical conditions forced the withdrawal of several patients who would otherwise have been likely to complete the SET. A similar drop-out rate was consistently observed across other exercise interventions in patients with IC, with figures quoted generally in the range of 15-25% and most cases attributable to medical conditions (115, 121, 122, 130).

An exception to this was Gelin et al(128), whose much larger drop-out rate (33%) was associated with a 12 month exercise intervention, with many participants citing too much commitment as the reason for withdrawal.

Of the studies that reported compliance data, the range of mean compliance was 73% (114) to 95% (236). The compliance rate of 85% (Group A) and 83% (Group B) reported in the RCT presented in this thesis is therefore considered to be acceptable.

Several factors are likely to be facilitators for the levels of retention and adherence data observed in the RCT presented in this thesis. Certainly a degree of selection bias exists at the initial recruitment phase, in which generally only the more motivated patients are likely to consent to participate. Supervised programs are known to have an advantage over home-based exercise (289) and it was noticed that many participants viewed the opportunity to exercise with a group of patients sharing similar demographics, as somewhat of a social occasion. This may well promote an increased level of motivation and positive attitude towards exercise. It was also noted that those participants who demonstrated a sense of self-efficacy, positive attitude and belief in the benefits of exercise, were the most compliant. Although in the present study this is simply an observation and there are no data to support this concept, it has been previously described (290).

The moderate intensity of the prescribed exercise may also contribute to the high levels of adherence to the SET. There is evidence suggesting enhanced exercise compliance in association with moderate intensity activity as opposed to high intensity exercise, particularly in those with limited exercise experience (291).

As knowledge regarding the optimal exercise prescription for patients with IC improves, it may be possible to tailor SET to meet the requirements of each individual, with the possibility that compliance will improve to an even higher level, as has been suggested by the ACSM (97). Regardless, the experience from the RCT presented in this thesis would certainly suggest that patients with IC who choose to

participate in SET are motivated to improve QoL and level of physical function and retention and adherence rates reflect this.

3.4.7 Quality of Life

Improvement in QoL is one of the most widely recognised benefits of exercise. This is probably multi-factorial and relates to improvements in cardio-respiratory fitness and health outcomes, increased social interaction and a positive dose-dependent relationship between physical activity and energy levels (292). It is therefore not surprising that irrespective of change in walking performance or exercise modality, the RCT presented in this thesis reported widespread and significant improvement in QoL. Despite these findings, it is interesting to note the contrast that exists in the literature with respect to QoL data. The QoL instrument utilised in this study (AUSVIQOL) (285) is unique in that it integrates both disease specific and generic QoL components, however, it has not previously been utilised in other similar trials investigating the impact of SET on QoL in patients with IC. Most literature reporting on the impact of a SET on QoL in patients with IC have used both disease specific instruments (e.g. Walking Impairment Questionnaire) and generic instruments (e.g. Medical Outcomes Study Short Form 36 (MOS-SF-36) which assesses both physical and mental health components of QoL using several sub-categories). Invariably, the Walking Impairment Questionnaire improves, likely reflecting walking performance, but such improvements are not always detected using generic instruments. Gardner et al (114, 130) and Savage et al (293) reported combined scores for each sub-category of the MOS-SF-36 and demonstrated no significant change. Regensteiner et al (124) and McDermott et al (122) used a similar method of reporting and did suggest a significant QoL improvement after an exercise intervention, while Kakkos et al (136) and Tsai et al (115) reported results for each sub-category of MOS-SF-36 and

demonstrated improvement in some areas and not others, with no consistent trend identified. While pleasingly, no groups have reported deterioration in QoL scores, the inconsistent findings of generic QoL instruments may be due to the fact that patients with IC often suffer multiple underlying co-morbidities and ambulatory dysfunction may be only one component to influence subjective health related QoL. Furthermore, QoL benefits of SET may lag behind improvements in walking performance. Long term collection of QoL data may therefore be warranted.

3.4.8 Conclusions

The quest for the most effective exercise regimen to maximise improvements in walking performance in patients with IC remains ongoing.

The study presented in this thesis was unable to confirm the hypothesis that a combined treadmill and resistance training program can achieve the equivalent or more significant gains in walking performance as a program of treadmill training alone. Several reasons have been proposed to explain this, including the possibility that in an attempt to successfully combine two exercise modalities the participants in the combination group were not exposed to a sufficient volume of either treadmill or resistance training to enable an adaptive response to either modality to occur. Furthermore variability in response to exercise may have negated the potential to observe a significant result. Although neither exercise regimen was superior in terms of walking performance, both groups displayed improvements in QoL and satisfactory levels of adherence to the intervention.

The following chapters describes the effect of the two exercise modalities on endothelial function and then on inflammatory markers in an effort to determine the

potential effects of the exercise modalities on a broad range of both clinically and biologically important outcomes.

Findings from Chapter 3 have been published in the European Journal of Vascular and Endovascular Surgery: *Delaney CL, Miller MD, Chataway TK, Spark JI. A randomised controlled trial of supervised exercise regimens and their impact on walking performance, skeletal muscle mass and calpain activity in patients with intermittent claudication. Eur J Vasc Endovasc Surg. 2014;47(3):304-310.*

CHAPTER 4: IMPACT OF THE INTERVENTION ON NON-INVASIVE MARKERS OF ENDOTHELIAL FUNCTION

4.1 Introduction

The vascular endothelium plays a key role in regulating inflammation, coagulation, vasomotor tone and smooth muscle cell proliferation (294). Endothelial dysfunction is a state of impairment of these regulatory functions and is one of the initial pathological processes of atherosclerosis (294). Endothelial dysfunction is characterised by an impaired endothelium dependent vasodilatory response induced by changes in flow or stimuli (54). As previously discussed (*See section 1.2.5* Assessment of endothelial function*)* it can be assessed by various techniques including FMD and RH-PAT. Nitric oxide is a potent vasodilatory agent secreted by the endothelium whose pleiotropic effects include the inhibition of both platelet aggregation and inflammation (54). Reduced levels of NO play a key role in the pathogenesis of ED and help to explain the association of ED with a pro-thrombotic and systemic pro-inflammatory state (54). The latter is evidenced by increased levels of inflammatory cytokines including IL-6 and TNF-α. Asymmetric dimethylarginine is an endogenous, competitive inhibitor of NO production, whose plasma levels have been shown to be elevated in patients with atherosclerosis (68). It may therefore contribute to the state of ED.

Clinically, the vasoconstrictive state of ED may worsen the anatomical stenosis of atherosclerotic plaque in patients with IC, contributing to more severe symptomatology (147). Exercise in healthy individuals has been shown to augment blood flow and intravascular shear stress, resulting in increased NO production and upregulation of eNOS activity, thus improving endothelial function. Whether or not

SET can induce similar improvement in patients with IC has not yet been elucidated, nor has the possibility that different exercise regimens may have a differing effect on endothelial function.

4.2 Aim

The aim of this chapter is to address RQ2: "Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant improvement in non-invasive markers of endothelial function (FMD, RH-PAT, NO and ADMA) compared to an exercise regimen consisting of treadmill training alone in patients with IC?"

4.3 Results

4.3.1 Participants

Data were collected from participants recruited and randomised to participate in the 12 week SET as described previously (*See section 3.3* Participants*).* With respect to FMD, NO and ADMA analyses, the sample size in each group for both per-protocol and ITT purposes is unchanged. Reactive Hyperemia Peripheral Arterial Tonometry technology was unavailable for the first six participants due to a delay in delivery of the equipment. Reactive Hyperemia Peripheral Arterial Tonometry data were therefore collected from 15 (10M, 5F) and 14 (11M, 3F) participants in Group A and B respectively. Given that two of the seven patients who withdrew from the SET were from the first six participants and both in Group B, per-protocol analysis for RH-PAT consisted of 12 (10M, 2F) participants in Group A and 11 (8M, 3F) participants in Group B.

All within and between group changes for the non-invasive markers of endothelial function are presented in *Table 5*.

4.3.2 Flow Mediated Dilatation (FMD)

There was no significant difference in baseline FMD between groups (P=0.398). Change in FMD from baseline to 12 weeks was not statistically significant between groups. There was no change in FMD from baseline to 12 weeks observed in either group.

4.3.3 Reactive-Hyperaemia Peripheral Arterial Tonometry (PAT)

There was no significant difference in baseline RHI between groups (P=.193). There was no change in RHI from baseline to 12 weeks observed in either group.

4.3.4 Nitric Oxide (NO)

There was no significant difference in baseline NO between groups (P=.205). With per protocol analysis, NO activity was significantly reduced as a result of the SET in Group A (P=.002) but not Group B. This also produced a statistically significant between group difference (P=.040). With ITT analysis, NO remained lower after intervention in Group A (P=.003) and not Group B, however, while there was a trend towards a significant difference between groups, this did not reach statistical significance (P=.066). No correlation was observed between baseline levels of NO and FMD (r = 0.02, P=0.93), RH-PAT (r = 0.12, P=0.55) or ADMA (r = 0.14, P=0.42).

4.3.5 Asymmetric Dimethylarginine (ADMA)

There was no significant difference in baseline ADMA between groups (P=.709). After the 12 week SET, ADMA remained virtually at baseline in Group A for both ITT and per-protocol analyses. In contrast, the level of ADMA was significantly reduced in Group B participants with ITT analysis (P=.028), while the reduction observed from per-protocol analysis was also significant (P=.05). These differences

were not statistically significant between groups, P=.193 and P=.236 for ITT and per-protocol analysis respectively.

4.3.6 Responders vs Non-Responders

There were 10 (67%) participants in Group A who demonstrated improvement in PFWD and 5 (42%) in Group B (*See Table 4*). Within each group, there was no significant change observed between responders and non-responders in FMD (Group A Responders: 0.38 (2.17) vs Non-responders: 0.89 (1.51), p=0.65 and Group B Responders: 0.11 (3.53) vs Non responders: -0.47 (4.29), p=0.81), RHI (Group A: -0.03 (0.89) vs 0.17 (0.43), p=0.72 and Group B: 0.23 (0.71) vs 0.08 (0.78), p=0.75), NO (Group A: -5.42 (6.49) vs -10.67 (8.95), p=0.21 and Group B: -3.65 (6.87) vs -2.12 (6.08), p=0.68) or ADMA (Group A: -0.02 (0.11) vs 0.01 (0.14), p=0.64 and Group B: -0.10 (0.15) vs 0.06 (0.09), p=0.53.

4.3.7 Summary of findings

These results have demonstrated that a 12 week exercise intervention consisting of either treadmill only exercise of a combination of treadmill and lower limb resistance exercises does not lead to improvements or deterioration in endothelial function as measured by non-invasive techniques including FMD and RH-PAT. In contrast, treadmill only training led to a significant reduction in serum NO level that was not detected in the combination training group, while the opposite was true with respect to serum ADMA which was significantly reduced in the combination group but not the treadmill only group. Furthermore, a positive response to the SET with respect to walking performance (PFWD) appears to be independent of endothelial function.

Table 5: Intention to treat and per protocol analysis demonstrating between and within group differences for those participating in either the 12 week treadmill only supervised exercise program or the 12 week treadmill and resistance training supervised exercise program: measures of endothelial function. Data presented as median (IQR) unless otherwise stated

Outcome	**Treadmill only (Group 1)**				**Treadmill and Resistance Training (Group 2)**				**Between groups**
	Baseline	**12 Weeks**	**Change**	**P-*value***	**Baseline**	**12 Weeks**	**Change**	**P-*value***	**P-*value***
Intention to treat									
Flow mediated dilatation, %	1.3 (0.7, 3.0)	1.3 (0.9, 4.6)	0.3 (-0.3, 1.9)	.281	2.4 (0.4, 5.0)	1.2 (0.1, 4.4)	-0.8 (-2.7, 0.8)	.382	.110
Reactive Hyperaemia Index	1.7 (1.6, 2.2)	2.1 (1.5, 2.4)	0 (-0.2, 0.5)	.824	1.6 (1.3, 1.9)	1.6 (1.4, 1.8)	0 (-0.2, 0.3)	.689	.769
Nitric Oxide, µmol/L[a]	15.0 (10.3)	8.3 (5.4)	-6.3. (7.4)	.003	11.2 (5.5)	9.1 (7.4)	-2.1 (5.5)	.138	.066
ADMA, µmol/L	0.58 (0.56, 0.67)	0.58 (0.51, 0.65)	0 (-0.07, 0.07)	.776	0.61 (0.56, 0.67)	0.56 (0.50, 0.63)	-0.03 (-0.15,0)	.028	.193
Per protocol									
Flow mediated dilatation, %	1.4 (1.0, 3.2)	1.4 (0.9, 4.9)	0.6 (-0.3, 2.1)	.281	2.9 (0.2, 5.0)	1.2 (0.1, 5.0)	-1.0 (-2.9, 0.43)	.272	.075
Reactive Hyperaemia Index	1.7 (1.6, 2.1)	2.1 (1.5, 2.3.)	0 (-0.3, 0.6)	.824	1.5 (1.1, 2.2)	1.6 (1.3, 1.7)	0 (-0.3, 0.3)	.906	.928
Nitric Oxide, µmol/L[a]	15.4 (10.5)	8.2 (5.6)	-7.2 (7.5)	.002	9.2 (3.6)	7.5 (7.1)	-1.7 (5.1)	.282	.040
ADMA, µmol/L	0.58 (0.56, 0.64)	0.57 (0.51, 0.64)	0.01 (-0.07, 0.08)	.776	0.65 (0.56, 0.68)	0.56 (0.49, 0.64)	-0.05 (-0.16, 0.01)	.050	.236

[a] Data presented as mean (SD); Abbreviations: ADMA: Asymmetric dimethyl arginine

4.4 Discussion

Augmentation and maintenance of endothelial function is associated with the prevention of CVD and is becoming increasingly recognised as an important beneficial effect of regular physical activity (109, 295, 296). Exercise induced intravascular shear stress has been demonstrated to up-regulate activity of NOS with a subsequent increase in the bioavailability of NO (65) and is proposed to be responsible for the improved endothelial function demonstrated in patients with CAD (109), hypertension (297) and indeed healthy individuals (98) after structured exercise programs.

It is therefore somewhat surprising that both exercise modalities undertaken in this study have not resulted in any clinically detectable improvement in endothelial function and in fact, reduced levels of NO observed in the treadmill only group may be associated with a potentially detrimental deterioration in endothelial function. This finding together with the potentially beneficial improvement in ADMA levels observed in the combination training group suggests that a combination training exercise regimen may be superior to a treadmill only exercise program with respect to impact on endothelial function.

Greater exposure to IRI in the treadmill only group may help to explain these findings, as the pathophysiological response to IRI is known to activate the endothelium, leading to a dysfunctional state, characterised by impaired NO bioavailability (194). While one would expect this impairment to be reflected in the non-invasive markers of endothelial function (FMD and RH-PAT), this was not the case. Reference to the literature to explain the rationale for this anomaly is limited by the scope and design of previous studies in this area.

4.4.1. Flow Mediated Dilatation (FMD)

Flow mediated dilatation has only been used to characterize endothelial function in four previous studies of a supervised exercise intervention in patients with IC and none of these have assessed an intervention comprising treadmill and resistance training (122, 231, 232, 298).

Table 6 compares and contrasts the four previous studies and the study presented in this thesis. There are obvious differences in the level of evidence amongst the studies, ranging from level 2 RCT's (122, 298) to level 4 pre-test/post-test studies (231, 232) and duration of the intervention ranges from six weeks (232) to six months (122, 231). The training modalities are similar across the groups with the exception of an additional resistance trained group in McDermott et al (122) and the combination based training regimen employed in the study presented in this thesis. The most striking feature of *Table 6* is the significant improvement in FMD reported following a treadmill training intervention in all four previous studies (122, 231, 232, 298). This is in contrast to the lack of change reported in FMD following a treadmill-based intervention in the study presented in this thesis. Interestingly, McDermott et al (122) also report no significant change in FMD following a lower-limb resistance training intervention compared to a usual care control group. This is in some way consistent with the lack of change reported following a combination-based intervention in the study reported in this thesis, suggesting that resistance training does not impact on FMD. Furthermore, a study of young men subjected to 12 weeks of whole body resistance training, demonstrated that the intervention had no impact on FMD (299).

A critical point of difference between the studies reported in *Table 6* is the technique employed to measure FMD. Brendle et al (231), McDermott et al (122)and Andreozzi et al (232) have all used the original technique for assessment of FMD, first described

by Celemajer in *The Lancet* in 1992(89). This involves the occlusive cuff being inflated around the upper arm, proximal to where the segment of brachial artery is being measured to assess for the degree of reactive hyperemia causing FMD. It has recently been demonstrated that while this technique results in a greater dilatation(300), it is not a solely an endothelium dependent NO-mediated response and is also reflective of a vascular smooth muscle myogenic and sympathetic response to ischaemia (233). In fact, NO-mediated responses contribute only about 40% of the vasodilatation observed with proximal cuff techniques (295).

Distal occlusion, in which the occlusive cuff is placed on the forearm distal to the segment of brachial artery being assessed, causes a lesser but more specifically endothelium dependent NO-mediated dilatation (233) and has recently become the recommended FMD method (53, 301, 302). For this reason, distal occlusion was the preferred method used in this study. The same technique was also used by Allen et al (298).

The results highlighted in *Table 6* clearly demonstrate that the three studies to have utilised the proximal cuff technique have all reported significant improvement in FMD following the SET (122, 231, 232). These studies also all report a larger magnitude of FMD compared with the study presented in this thesis and Allen et al (298). It is possible that the observed improvements in FMD reported in these three previous studies using proximal occlusion technique may be associated with improvements in myogenic and sympathetic ischaemic conditioning, both of which are independent of the vascular endothelium and were therefore not identified in the study presented in this thesis due to the use of the distal occlusion technique.

Table 6: Summary of studies of an exercise intervention in patients with IC reporting Flow Mediated Dilatation as an outcome.

Author, year	*Type of study*	*Sample size, n*	*Training modality*	*Duration of SEP*	*Technique for FMD*	*Analysis of data*	*Results*	*Bias*
Andreozzi, 2007(232)	pre-test/post-test	22	Treadmill to 60-70% PFWD	6/52, three sessions/wk, daily distance 1-2km	Proximal cuff	Manual	7.6% (2.94) to 10.3 (4.04), P<0.01 [Mean (SD)]	N/A
Brendle, 2001(231)	pre-test/post-test	19	Treadmill to near maximal claudication pain	6/12, three sessions/wk, 15-30mins walking per session	Proximal cuff	Manual	4.81% (0.82) to 7.97% (1.03), P<0.005 [Mean (SD)]	N/A
McDermott, 2009(122)	RCT	37 treadmill only SEP (Group 1); 36 lower limb resistance SEP (Group 2); 28 usual care control (Group 3)	Treadmill to near maximal claudication pain OR lower limb resistance as prescribed by certified trainer	6/12, three sessions/wk, 15-40mins exercise per session	Proximal cuff	Manual	Group 1: 5.54% (3.09, 6.87) to 5.39% (3.27, 8.16), P=0.02 vs Group 3. Group 2: 4.89% (2.59, 9.24) to 6.13% (2.20, 8.37), P=0.23 vs Group 3. Group 3: 5.97% (3.80, 8.91) to 5.28% (3.60, 7.44). [Median (IQR)]	Not mentioned
Allen, 2010(298)	RCT	18 usual care control, 15 in intervention	Treadmill to moderately severe claudication pain	3/12, three sessions/wk	Distal cuff	Manual	Control: 2.1% (0.5) to 3.2% (0.8), P>0.05. Intervention: 2.4% (0.8) to 4.3% (0.6), P<0.05 [Mean (SD)]	Not mentioned
Delaney, 2015	RCT	18 treadmill only SEP (Group 1); 17 interval treadmill and lower limb resistance SEP (Group 2)	As described in sections 2.5.1.1 and 2.5.1.2 of this thesis	3/12, two sessions/wk, one hour duration per session	Distal cuff	Automatic software	Group 1: 1.3% (0.7, 3.0) to 1.3% (0.9 , 4.6), P=.28. Group 2: 2.4% (0.4, 5.0) to 1.2% (0.1, 4.4), P=0.11 [Median (IQR)]	No

Interestingly, Allen et al (298) also utilised the distal occlusion technique and demonstrated a significant improvement in FMD after subjecting patients with IC to three months of supervised treadmill based training. This study implemented a very similar protocol in an equivalent sample size of patients with demographics comparable to this study. Despite this, there are a couple of reasons why results of the two studies are different. Firstly, it was unclear from Allen et al (298) whether or not assessors were blinded to allocation, raising the possibility of bias. Secondly, normality testing in the study presented in this thesis demonstrated that data relating to FMD were distributed in a non-parametric manner and results were subsequently presented as median (IQR). It seems that normality testing has not been performed in Allen et al (298) who have instead assumed data to be normally distributed and reported as mean (SD). Results of Allen et al (298) may therefore be reflective of inaccurate statistical methods.

It is possible then, that 12 weeks of SET in the patient cohort presented in this thesis simply did not or will not impact on FMD and improvement in endothelial function is not a mechanism by which SET augments walking performance in patients with IC. It is also possible that in the study presented in this thesis, there may be improvement in NO-mediated FMD that is masked by the variability associated with technique.

Assessment of FMD is reliant on the quality of images and measurements and is therefore highly operator dependent. It has been recommended that 100 supervised scans and measurements are performed to achieve a level of competency required to reliably measure FMD as an outcome measure of an intervention (303). Also relevant is the issue of physiological variability leading to high inter-day variability, with reported CV's for FMD reproducibility ranging from 10.3% to >50% (304). Intra-individual variation in FMD has been attributed to local, systemic and environmental

factors including endogenous hormone secretion blood pressure, vessel compliance, autonomic nervous system activity and temperature (304, 305).

Previous unpublished work from the candidate has demonstrated that despite ensuring environmental conditions were well controlled for, as is the case in the study presented in this thesis, this alone was not sufficient to overcome the problem of inter-day variability and a CV of 32.9% was observed. Although this falls within the previously reported range and is therefore acceptable, the consequence is either that a larger sample size is required to provide sufficient power to detect any significant change in FMD or that the small effect size associated with the intervention is masked by the variability of the FMD response and therefore remains undetected.

4.4.2 Reactive Hyperemia Peripheral Arterial Tonometry (RH-PAT)

The EndoPAT device uses a proximal cuff occlusion technique to measure RH-PAT and was designed for use in a primary care setting, based on its intention to eliminate the operator-dependent variability associated with FMD and the concept that proximal cuff occlusion, through its multifactorial assessment of myogenic, sympathetic and NO mediated response to ischaemia, is able to provide information that is more predictive of cardiovascular events than simply measuring NO dependent endothelial responses alone using distal cuff occlusion techniques(91). Despite this, previous unpublished work undertaken by the candidate prior to the commencement of the study presented in this thesis demonstrated the inter-day reproducibility of RH-PAT to be 19.3%. This was an obvious improvement compared with the CV of 32.9% associated with FMD and this is consistent with previous work to assess the impact of exercise on RH-PAT in patients with metabolic syndrome, from which it was concluded that magnitude of effect on RH-PAT ratio must be greater than 20% to eliminate possible measurement errors (92).

This study is unique in that it is the first to assess the impact of SET in patients with IC on the RH-PAT ratio. While there is no literature for direct comparison, there are several studies to suggest a correlation between FMD arising from proximal occlusion techniques and RH-PAT (90-92, 306). It would therefore seem reasonable to suggest an improvement in RH-PAT would be observed, consistent with the three previously mentioned studies *(See Table 6)* that used *pr*oximal cuff techniques and demonstrated an improvement in FMD in patients with IC following SET. It should be noted however, that RH-PAT measures the response of resistive digital vessels to reactive hyperemia (295). These consist of micro and macro-vasculature with a significant role played by arterio-venous anastomoses that are primarily regulated by the sympathetic nervous system (295). Flow mediated dilatation measures the hyperaemic response in large conduit vessels. Of the studies to demonstrate correlation between proximal cuff FMD and RH-PAT, study populations ranged from patients with CAD (91) to systemic lupus erythematosus (93) and healthy individuals(307), with no consideration given to patients with PAD. Microvascular changes are known to be associated with PAD, potentially impacting on any relationship that may be expected between proximal cuff FMD and RH-PAT, as well as blunting any impact of exercise on RH-PAT. This may in some way explain why an improvement in RH-PAT was not observed in the study presented in this thesis, as may have been expected on the basis of previous work to have utilised proximal cuff occlusion techniques to measure FMD. Instead, the findings for RH-PAT in the study presented in this thesis support the findings of the distal occlusion FMD technique that was utilised, that is, there is no impact on endothelial function from 12 weeks of SET as measured by non-invasive techniques.

In addition to the physiology of the circulations being tested, as described above, there are other reasons to explain why an improvement in endothelial function as measured by RH-PAT was not observed in the study presented in this thesis. Firstly, like with FMD, it is possible that exercise in our patient cohort simply did not impact on endothelial function at all. Secondly, the effect size may have been within the 20% required to overcome measurement error.

A strength of RH-PAT assessment is its validation as a screening tool and an RH-PAT index of ≤1.67 is highly sensitive and specific for the diagnosis of ED (308). This brings to light an important concept relevant to both the FMD and RH-PAT results presented in this thesis. As described above, there are no studies with which to compare the impact of SET on RH-PAT, however, the baseline levels of RH-PAT in both Group A (1.7) and Group B (1.6) are effectively consistent with a diagnosis of ED as according to RH-PAT criteria. Similarly, although there are no established cut-offs for ED in association with FMD, when compared to the baseline value presented in Allen et al(298), the other study in *Table 6* to utilise the distal cuff occlusion technique, the baseline FMD for participants in Group A of the study presented in this thesis, is lower. It is possible then, that the magnitude of ED in the sample of participants in the study presented in this thesis means that the prescribed SET was unable to improve endothelial function manifesting as a change in FMD or RH-PAT. It may be that a higher frequency or longer duration of SET is/are required to demonstrate an improvement in either FMD or RH-PAT. It may also be that biological rather than clinical markers of endothelial function are more sensitive, such as was observed with NO, where the magnitude of change was large enough that the differences between groups were observed on per-protocol analysis.

4.4.3 Nitric Oxide (NO)

The link between NO bioavailability and endothelial function is indisputable but despite this, there is a paucity of literature reporting NO values as a surrogate measure of endothelial function and only two studies to assess the impact of exercise training on either NO levels or metabolites of NO (nitrite and nitrate) in patients with IC (234, 298). Allen et al (298) demonstrated a significant improvement in acute post-exercise plasma nitrite flux after three months of supervised treadmill based exercise in patients with IC associated with a significant mean improvement in PFWD. Interestingly and perhaps not unexpectedly given the link between NO and distal occlusion FMD, this correlated with an improvement in pre-exercise FMD, following the three month SET, using a distal cuff occlusion technique, however, pre-exercise plasma nitrite levels were not influenced by the SET. Findings from Arosio et al (234) provide some support to this work from Allen et al (298), by demonstrating a significant increase in resting urinary nitrite/nitrate levels after 14 days of SET, suggested to arise from an increased level of systemic NO production in association with exercise.

The results presented in this thesis are in contrast to these previous studies, particularly with respect to participants in the treadmill training group, in whom, 12 weeks of SET led to a significant reduction in resting plasma NO from baseline. A non-significant reduction in plasma NO was also observed in those undertaking combination training.

Before discussing potential reasons for the observed results and the conflict that exists between the study presented in this thesis and previous work, it is important to consider that NO production may be driven not only eNOS (64), but also iNOS whose activation is suggested to be induced by inflammatory mediated pathways and may be

associated with acute high levels of NO production with subsequent oxidative free radical formation and cell toxicity, potentially contributing to IRI (309). With respect to results from this study, the observed reduction in NO levels, particularly for those undertaking treadmill only training, is likely to reflect a detrimental attenuation of eNOS activity driven by repeated bouts of IRI and associated release of pro-inflammatory and pro-oxidative agents that are damaging to the endothelium, thus impairing the role of eNOS. Exposure to IRI is expected to be reduced in the combination training group, which may explain the difference observed between the two groups.

It could be argued that such results are actually associated with a dampening of iNOS activity, however, this is unlikely given that the results of *Chapter 5 – Impact of the intervention on inflammatory response and burden* demonstrates no clear trend towards a pro- or anti-inflammatory response to 12 weeks of SET. Furthermore, it seems that iNOS, unlike eNOS is not constitutively expressed and its activity only contributes to the acute inflammatory response rather than chronic resting levels measured in this study, which are more reflective of the general health of the endothelium.

Such a concept however, could be used to explain the acute increase in post-exercise plasma nitrite flux observed in Allen et al(298). In these patients who significantly improved their walking performance as a result of the SET, the duration of IRI is likely to have lengthened correspondingly, potentially leading to increased activation of iNOS. Alternatively, as has been proposed by the Allen et al(298), the findings may represent an improved capacity of the endothelium via eNOS to produce NO in response to a shear stress stimulus, either exercise or reactive hyperemia as is demonstrated by an associated improvement in FMD. While this seems the more

likely explanation and is supported by evidence to suggest that nitrite supplementation enhances exercise performance in patients with PAD (310), to confirm this would require flow cytometric assessment of iNOS versus eNOS subtypes.

The study presented in this thesis did not measure nitrite flux immediately post exercise and for this reason, comparisons cannot be drawn directly with this component of the Allen et al (298) study. It could be speculated however that an improvement in both FMD and post-exercise nitrite flux would translate to an improvement in resting NO bioavailability in Allen et al (298), however, this was not the case, as resting nitrite levels were not influenced by 12 weeks of SET. This suggests that an improved ability of the endothelium to respond to an acute stimulus such as exercise rather than an increase in constitutively expressed eNOS may be a mechanism of improved walking performance in patients with IC.

In contrast to this, as was mentioned previously, the findings of the study presented in this thesis suggest that IRI is detrimental to the endothelium and manifests as a reduction in resting NO levels.

Several differences between the two studies are also worthy of consideration and may contribute to the conflicting results. The study presented in this thesis used an ELISA based method in which the well-validated Griess reaction was used to measure NO concentration based on the contribution of both nitrite and nitrate ions. In contrast, a chemiluminescence technique was used by Allen et al (298), specifically measuring nitrite levels but not nitrate. This is important because NO and nitrite are rapidly oxidised to nitrate in whole blood and the half-life of nitrite in human blood is approximately 110 seconds, compared with the more stable nitrate, whose circulating half life is 5-8 hours. Neglecting to measure nitrate may therefore be a critical

omission in Allen et al (298) as nitrite levels alone are unlikely to provide an accurate representation of total endogenous NO production.

Furthermore, Jarvisalo et al (305) suggests that levels of nitrite and nitrate are closely dependent on dietary intake and are therefore not specific markers for NO degradation. Although in the study presented in this thesis, it was found that dietary intake has been minimally influenced by the 12 week SET in patients with IC (*See Chapter 6)*, the technique utilised for this is representative of trends in intake across a period of days to weeks rather than acute intake 12-24 hours before blood sampling which is the period most likely to impact on nitrite and nitrate levels. In Allen et al (298), no mention has been made of controlling for dietary intake. This concept therefore has the potential to confound results from both studies and may contribute to the inconsistency in the results observed.

Only Arosio et al (234) have controlled diet in order to avoid the influence of food on their urinary measurement of nitrite and nitrate. Their results however, are in further contrast to those of the study presented in this thesis and Allen et al (298), with Arosio et al (234)reporting an increase in resting urinary excretion of nitrites and nitrates after only 14 days of SET in patients with IC. This is likely to reflect improved basal endothelial NO production rather than the improvement in NO production in response to an acute stimulus suggested by the results of Allen et al (298). It is likely that eliminating diet as a variable has allowed Arosio et al (234) to generate more accurate results with respect to resting NO production compared with the study presented in this thesis and that of Allen et al (298), however, it seems unlikely that this alone can account for the large variation in results among the studies.

The exercise protocol used by Arosio et al (234) incorporated treadmill training, resistance exercises, cycling and other dynamic exercises such as lunges and squats. This is more reflective of the combination training group of the study presented in this thesis, in which it is speculated that less exposure to IRI resulted in a non-significant change to resting NO levels. In addition, while the 14 day duration of exercise, implemented by Arosio et al, seems short relative to the three month programs of the study presented in this thesis and Allen et al (298), Arosio et al (234) has used 14 days of consecutive exercise which would equate to the volume of training achieved in seven weeks of the SET in the study presented in this thesis. It is possible then that the positive results of Arosio et al (234)with respect to resting NO production are reflective of a short, high-volume exercise program with limited exposure to IRI. This may enable greater improvements to the basal health of the endothelium rather than just an adaptive response to acute stimuli.

Further beneficial effects of such a program are demonstrated by a significant improvement in walking performance and an anti-inflammatory effect manifesting as reduced neutrophil adhesion. It is important to note however, that the observed increase in urinary nitrite and nitrate excretion and improvement in neutrophil adhesion returned to baseline levels within 7-10 days following the cessation of exercise. Whether or not a longer duration of the same high frequency and high volume SET would enable a more sustained beneficial effect remains to be seen.

This concept of transient versus prolonged effect should also be applied to other markers of endothelial function as well as all variables used to assess the impact of SET on clinical, systemic and local biological responses in patients with IC. It is possible for example, that the observed difference in FMD reported in this thesis compared with Allen et al (298), as discussed previously, is due to differences in

timing after completion of the SET. The protocol for the study presented in this thesis required outcome measurements to be performed within one week after the completion of SET, at which point, results from Arosio et al (234) would suggest that any transient effect may have worn off. Work from Allen et al (298)did not state a time frame during which follow-up measurements were performed. Potentially, the study presented in this thesis may have observed different results if all patients had undergone follow-up testing the day after completion of the SET. The same may hold true for RH-PAT measurements.

Given the known close association between NO bioavailability and endothelial function and the fact that NO concentration has been proposed as a surrogate marker of endothelial function, it would be expected that measurement of such parameters would display a degree of correlation. In particular, FMD using distal cuff occlusion techniques are largely NO dependent. Despite this, in the study presented in this thesis no correlation between baseline levels of NO and FMD was observed. While Allen et al (298) report a significant correlation between the two markers, results of the study presented in this thesis are supported by Jarvisalo et al (305) whose study using distal occlusion FMD in healthy young men also failed to demonstrate a correlation with NO. This was largely attributed to the impact of dietary intake on nitrite and nitrate levels rather than the influence of endothelium derived NO bioavailability. It must also be considered that FMD represents an endothelial response to an acute stimulus rather than the resting level of endothelial function represented by NO levels. Alternatively, in the case of the study presented in this thesis, it could be speculated that endothelial function reaches a level below which it is so dysfunctional that further changes in NO levels have little influence on function.

This concept of diet and acute versus chronic health of the endothelium could also be used to explain the lack of observed correlation between NO and RH-PAT, although given that RH-PAT values are only partially influenced by NO, this finding is not really surprising. No other studies have previously investigated such a relationship.

4.4.4 Asymmetric Dimethyl Arginine (ADMA)

An endogenous, competitive inhibitor of the L-arginine required for synthesis of NO, ADMA has been shown to be present at raised levels in patients with PAD and has been proposed as an independent risk factor for the development of ED (54, 68). It therefore has the potential to provide insight into possible mechanisms through which exercise in patients with IC impacts on the endothelium. Despite this, there exists a paucity of literature in the area, with only Schlager et al (311) affording any attention to such a concept.

Schlager et al (311) reported a significant reduction in plasma ADMA levels in patients with IC randomised to undertake six months of treadmill based SET compared with those randomised to best medical therapy only. The SET was also associated with a significant improvement in PFWD in these patients. The results presented in this thesis are somewhat in contrast to this, demonstrating that three months of supervised treadmill based training did not impact on ADMA levels, however, a significant improvement in ADMA was observed following a combination training regimen.

If ADMA was the sole regulator of NO synthesis, a relationship between both ADMA and NO would be expected. While NO levels were not influenced by exercise in the combination training group ADMA levels were found to decrease and the absence of an increase in ADMA level in the treadmill only group was not consistent with the

reported reduction in NO. Furthermore, the lack of observed correlation between the two certainly supports the fact that the mechanism underlying NO synthesis is multi-factorial potentially with some confounding influence from the previously discussed impact of diet on the measured metabolites of NO. In fact, it is widely recognised that a vast interplay of mechanisms contribute to the synthesis of NO, including gene expression of enzymes responsible for arginine and NOS synthesis as well as the presence of co-factors such as tetrahydrobiopterin (BH_4) (312).

Importantly, the results presented in this thesis suggest some benefit to endothelial function through the reduction of ADMA as a result of 12 weeks of combination based SET. Although this was not significantly better than the treadmill training group, a trend was certainly evident and this lack of observed significance may simply represent a type II statistical error. The mechanism underlying such an improvement is not entirely clear, however, systemic inflammation has been linked to high plasma ADMA levels (313) and it is therefore possible that exercise in the context of less exposure to IRI in the combination training group is responsible for such an improvement, although results presented in *Chapter 5* suggest no clear trend towards a pro or anti-inflammatory response to the SET in either group. Regardless, the clinical impact of such a finding remains unknown as it has not yet translated into a clinically detectable improvement in endothelial function as measured by FMD or RH-PAT.

With respect to the conflict between the ADMA results of patients with IC undertaking treadmill based training in the study presented in this thesis and those of Schlager et al(311), an obvious difference is the six month SET in Schlager et al(311) compared with the three month SET in the study presented in this thesis, however, this becomes irrelevant when we consider that Schlager et al(311) also reported

improved ADMA levels at the midway point (three months) of her study. Perhaps a better explanation for the difference in results between the two studies is the fact that disease severity was not well matched between the studies, with over 80% of patients recruited by Schlager et al(311) categorised as having severe claudication (Rutherford Category 3) compared with 97% of patients in the current study who experienced mild-moderate IC (Rutherford Category 1-2). This was reflected in a higher baseline level of ADMA in Schlager et al (311) compared with the study presented in this thesis, despite use of the same commercially available ADMA ELISA kit. Further work is certainly required to better understand the influence of exercise on ADMA in patients with IC and the potential impact of this on endothelial function and subsequently improvements in walking performance.

Interestingly, Schlager et al (311) also measured endothelial progenitor cells which are bone-marrow derived cells that have been shown to augment angiogenesis and promote repair of a damaged endothelium. Their levels were found to increase after six months of SET, potentially contributing to an improvement in endothelial health and cardiovascular risk profile. They may therefore represent a novel strategy for the treatment and prevention of PAD in the future.

4.4.5 Overview of endothelial function

Based on previous work investigating the role of exercise on the endothelium across a variety of demographics ranging from healthy individuals to patients with severe cardiac pathology, it was expected that improvements in FMD associated with an increase in NO bioavailability, as well as improved RH-PAT values and a reduction in levels of the inhibitory molecule ADMA would be observed in the current study that administered 12 weeks of SET to patients with IC.

There is however, limited literature discussing the impact of exercise in patients with IC and the influence that exercise induced IRI is likely to have on the endothelial function of these patients. The findings of this chapter hence provides an original contribution to the evidence base in this area of study.

The results of the study presented in this thesis would suggest that a combination based exercise program may be superior to the current gold-standard treadmill training regimens with respect to endothelial function, however, the small sample size and potential limitations imposed by the study power mean that the evidence remains limited. Nitric oxide, the potent vasodilatory mediator released by a healthy endothelium whose levels are known to be reduced in a dysfunctional endothelium, was found to be significantly reduced in the treadmill training group after 12 weeks of SET, representing a detrimental impact of the treadmill training. In contrast, a positive effect of combination training was the significant reduction in ADMA levels. These findings may represent a reduced exposure to IRI as a result of combination training compared with treadmill training. Despite this, the suggestion that combination training maybe superior to treadmill training with respect to endothelial function remains speculative as the change in ADMA level was not statistically significantly different between groups.

Furthermore, the clinical significance of such findings is unclear as they did not translate to changes in FMD or RH-PAT which are proposed as surrogate markers to represent the overall health of the endothelium and have been used to predict the onset of future CVD events. Also, given that improvement in endothelial function has been proposed as a key mechanism to explain improvement in walking performance, one would expect participants in the combination training group to have improved their walking performance to a greater extent than the treadmill training group, however,

this was not the case. In fact, the lack of difference between markers of endothelial function in responders and non-responders to SET challenges this theory that improvement in endothelial function is a mechanism by which SET produces improvement in walking performance.

While an understanding of the role of the endothelium has greatly evolved in recent years, it is clear that its function is multi-factorial and influenced by an array of regulatory and inhibitory mechanisms, all of which contribute to make accurate and reliable assessment of endothelial function challenging. This is highlighted by the contrasting results that have been reported by the few studies, including the one presented in this thesis, which have focused on the impact of SET on endothelial function in patients with IC.

This may mean that it is not possible to rely solely on one technique to provide an accurate representation of the overall health of the endothelium. Rather, all available techniques could be used in concert to enable more accurate analysis of the impact of interventions such as SET on the endothelium.

Furthermore, larger and longer term studies are required to not only improve the power of studies in an attempt to overcome the known daily variation in FMD and RH-PAT values, but also to assess the duration of any observed effects of SET on the endothelium and whether or not exercise training promotes adaptation of the endothelium only to an acute stimulus or to the underlying basal health of the endothelium.

Findings from Chapter 4 have been published in Vascular: *Delaney CL, Miller MD, Allan RB, Spark JI. The impact of different supervised exercise regimens on endothelial function in patients with intermittent claudication. Vascular, 1708538114558329, first published on November 18, 2014.*

CHAPTER 5: RESULTS – IMPACT OF THE INTERVENTION ON INFLAMMATORY RESPONSE AND BURDEN

5.1 Introduction

An acute bout of exercise is known to precipitate an inflammatory response (190), while PAD is associated with a raised systemic inflammatory burden which may help to explain the increased rate of cardiovascular related morbidity and mortality in this group of patients (192, 314). Concerns have been raised that due to ischaemia-reperfusion responses, exercise in patients with IC may actually exacerbate the inflammatory burden and subsequently worsen the cardiovascular risk profile of these patients (314). Despite this, the evidence regarding the impact of exercise in patients with IC on response of inflammatory markers is poor and conflicting. Long-term exercise training in healthy people seems to have beneficial effects on the inflammatory response (190). In patients with IC however, it is unknown whether exercise is pro-inflammatory or anti-inflammatory. Furthermore, it is unclear whether long-term exercise produces an adaptive response to the initial inflammatory response or whether certain exercise regimens may invoke less of an inflammatory burden than others. Lower limb resistance training has the benefit of potentially improving SMM, muscle strength and functional ability, without inducing an IRI, therefore potentially minimising the systemic inflammatory burden.

5.2 Aims

The aim of this chapter is to address RQ3: “Does 12 weeks of SET consisting of interval treadmill and lower limb resistance training impact differently on systemic

inflammatory response and burden than an exercise regimen consisting of treadmill training alone in patients with IC?"

5.3 Results

5.3.1 Participants

Non-specific markers of inflammation were used to broadly characterise the systemic inflammatory burden before and after the exercise intervention and include CRP, neutrophils, lymphocytes, neutrophil-lymphocyte ratio (NLR) and homocysteine. Data for ITT and per-protocol analysis were therefore collected on the same cohort of patients as those described previously (*See section 3.3* Participants*).*

The more specific pro and anti-inflammatory cytokines were assessed immediately before exercise and immediately after exercise in week one and week 12 of the SET. For this reason, data were not able to be collected for ITT analysis, with the exception of the one patient from Group B whose non-compliance was the reason for unsatisfactory completion of the SET. The number of participants whose data were available for per-protocol analysis remained the same as described previously (*See section 3.3* Participants*).*

All within and between group changes for the non-specific markers of inflammation (*See sections 5.3.2* Neutrophils, Lymphocytes and Neutrophil-Lymphocyte Ratio (NLR) *and 5.3.3* Homocysteine and C-Reactive Protein (CRP)*)* are presented in *Table 7*.

All within and between group changes for the pro- and anti-inflammatory cytokines (*See sections 5.3.4* Interleukin 1b (IL-1β) *to 5.3.14* Neutrophil Elastase (NE)*)* are

presented in *Table 8* while *Table 9* details the accepted reference range of cytokines presented in this thesis and the role they play in the inflammatory response.

5.3.2 Neutrophils, Lymphocytes and Neutrophil-Lymphocyte Ratio (NLR)

After 12 weeks of SET, there was no significant change in neutrophil levels in either Group A or B with both per-protocol and ITT analysis. Lymphocyte levels were stable in Group A, however, Group B exhibited a trend towards a reduction in lymphocytes with ITT analysis which became significant when analysed per-protocol and resulted in a near significant difference in change between the two groups. Not surprisingly therefore, a trend towards an increase in NLR was observed in Group B but not Group A. In both groups, the median values for neutrophils and lymphocytes were within the normal laboratory reference range (Neutrophils - 1.80-7.50 $x10^9$/L, Lymphocytes – 1.0-3.0 $x10^9$/L).

5.3.3 Homocysteine and C-Reactive Protein (CRP)

Homocysteine levels increased significantly in Group A resulting in a median value post 12 weeks of SET that is greater than the quoted laboratory reference range of 4-14umol/L. A non-significant increase in homocysteine was observed in Group B but the magnitude of increase was significantly different between the groups.

An increase in CRP was observed in Group A with a corresponding decrease in Group B. In each case, however, the change was not significant, nor was the difference between the groups.

5.3.4 Interleukin 1b (IL-1β)

At baseline, 7/17 (41%) participants in Group A and 6/16 (38%) participants in Group B had undetectable levels of IL-1β. There was a significant decrease in IL-1β levels in Group B noted after an acute bout of exercise from the first exercise session to the last

session of the 12 week SET. This change was not significantly different to that observed in Group A and there were no other statistically significant changes observed in IL-1β levels in either group (*Table 8*).

5.3.5 Interleukin 2 (IL-2)

Median values at each time-point in Group A and B are within the normal range (0-5pg/ml). No significant change either within or between groups was observed in IL-2 following an acute bout of exercise or after 12 weeks of SET (*Table 8*).

5.3.6 Interleukin 6 (IL-6)

Median values at each time-point in Group A and B are within the normal range (0.01-11.5pg/ml). A strongly significant increase in IL-6 level was observed following an acute bout of exercise at the end of 12 weeks of SET in Group B. There were no other statistically significant changes observed in IL-6 levels in either group (*Table 8*).

5.3.7 Interleukin 7 (IL-7)

At baseline, 15/17 (88%) of participants in Group A and 12/16 (75%) of participants in Group B had undetectable serum levels of IL-7. Of those who completed the 12 week SET, 11/15 (73%) participants in Group A and 8/12 (67%) in Group B still had undetectable levels. No significant change either within or between groups was observed in IL-7 following an acute bout of exercise or after the SET (*Table 8*).

Table 7: Comparison of baseline, 12 week and absolute change for outcomes between participants in the treadmill only supervised exercise training and the combined treadmill and resistance exercise training. Data presented as mean (SD): Non-specific markers of inflammation

Outcome	**Treadmill only**				**Treadmill and Resistance Training**				**Between groups P-*value***
	Baseline	12 Weeks	Change	P-*value*	Baseline	12 Weeks	Change	P-*value*	
Intention to treat[a]									
Homocysteine, umol/L	11.99 (2.51)	15.54 (4.32)	3.55 (2.98)	***0.003***	13.72 (3.97)	14.68 (4.69)	0.96 (3.06)	0.280	***0.049***
Neutrophils, $x10^9$/L	4.36 (1.46)	4.41 (1.21)	0.05 (1.71)	***0.876***	4.55 (1.77)	4.97 (1.76)	0.42 (1.50)	0.333	***0.272***
Lymphocytes, $x10^9$/L	1.68 (0.68)	1.68 (0.65)	0.00 (0.32)	***0.978***	1.91 (0.93)	1.65 (0.62)	-0.26 (0.46)	0.061	***0.097***
Neutrophil Lymphocyte ratio	3.05 (1.97)	2.98 (1.32)	0.07 (0.95)	***0.711***	2.82 (1.61)	3.35 (1.52)	0.53 (1.04)	0.088	***0.087***
C-Reactive Protein, mg/L	2.70 (1.97)	3.63 (2.46)	0.93 (2.27)	***0.185***	4.15 (3.92)	3.41 (2.77)	-0.74 (2.81)	0.361	***0.118***

Per protocol

Homocysteine, umol/L	11.99 (2.51)	15.54 (4.32)	3.55 (2.98)	***0.003***	13.76 (4.15)	14.70 (4.90)	0.94 (3.20)	0.470	***0.056***
Neutrophils, $x10^9$/L	4.36 (1.46)	4.41 (1.21)	0.05 (1.71)	***0.876***	4.66 (1.80)	4.99 (1.84)	0.33 (1.53)	0.470	***0.610***
Lymphocytes, $x10^9$/L	1.68 (0.68)	1.68 (0.65)	0.00 (0.32)	***0.978***	1.89 (0.97)	1.59 (0.60)	-0.30 (0.46)	0.042	***0.066***
Neutrophil Lymphocyte Ratio	3.05 (1.97)	2.98 (1.32)	0.07 (0.95)	***0.711***	2.93 (1.63)	3.47 (1.53)	0.54 (1.08)	0.113	***0.103***
C-Reactive Protein, mg/L	2.70 (1.97)	3.63 (2.46)	0.93 (2.27)	***0.185***	4.34 (4.03)	3.37 (2.88)	-0.97 (2.81)	0.257	***0.083***

[a]Five and three participants in Group A and B respectively did not complete the study. Of these, three and two returned for follow-up assessment and hence contributed data for ITT purposes. Two and one did not return for a follow-up and hence had their baseline data carried forward to enable ITT analysis.

5.3.8 Interleukin 8 (IL-8)

Median values at each time-point in Group A and B are within the normal range (0-18pg/ml). In Group B a significant increase in IL-8 level was observed following an acute bout of exercise at the end of 12 weeks of SET in Group B. There were no other statistically significant changes observed in IL-8 levels in either group (*Table 8*).

5.3.9 Interleukin 10 (IL-10)

The median values in both Groups A and B at all time points are greater than the normal range (<5.0pg/ml). No significant change either within or between groups was observed in IL-10 following an acute bout of exercise or after the SET (*Table 8*).

5.3.10 Interleukin 12 (IL-12)

The median values in both Groups A and B at all time points are less than the normal range (35-182pg/ml). At baseline, 12/17 (71%) and 7/16 (44%) of participants in Group A and B respectively were found to have undetectable levels of IL-12 in their serum. A statistically significant reduction of IL-12 was observed after an acute bout of exercise at the end of the 12 week SET in Group A. There were no other statistically significant changes observed in IL-12 levels in either group (*Table 8*).

5.3.11 Interferon-gamma (IFN-γ)

Median values at each time-point in Group A and B are within the normal range (0-20pg/ml). A statistically significant reduction of IFN-γ was observed after an acute bout of exercise at the end of the 12 week SET in Group A. There were no other statistically significant changes observed in IFN-γ levels in either group (*Table 8*).

5.3.12 Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

Median values at each time-point in Group A and B are consistent with the normal range (<2.0pg/ml). A significant increase in GM-CSF level was observed following

an acute bout of exercise at the commencement of the 12 week SET in Group A. There were no other statistically significant changes observed in GM-CSF levels in either group (*Table 8*).

5.3.13 Tumour Necrosis Factor-alpha (TNF-α)

The median values in both Groups A and B at all time points are higher than the normal range (detectable in <15% of elderly humans) and 100% of participants in each group recorded a detectable level of TNF-α. No significant change either within or between groups was observed in TNF-α following an acute bout of exercise or after the SET (*Table 8*).

5.3.14 Neutrophil Elastase (NE)

In Group A, all median values observed for NE are greater than the normal range (<200ng/ml). Although not statistically significantly less than Group A, the baseline value for NE in Group B is within the normal range, however, after the initial bout of acute exercise it is found to increase to abnormal levels. At the completion of the SET both pre and post-exercise values of NE are greater than normal. Within both groups, an observed increase in NE levels after the initial acute bout of exercise is significant. In Group B, the increase in pre-exercise NE level from the first to the last exercise session of the 12 week SET is strongly significant. In Group A, the post-exercise NE level is non-significantly decreased from the first to the last exercise session while the corresponding values in Group B are non-significantly increased. The resultant net effect of this is a significant change between the two groups (*Table 8*).

Table 8: Comparison of cytokine values at rest and following an acute bout of exercise at baseline and 12 weeks, between participants in the treadmill only supervised exercise training and the combined treadmill and resistance exercise training. Results are presented as mean (SD).

	Treadmill only (Group 1)				**Treadmill and Resistance Training (Group 2)**				**Between**
	Baseline	12 Weeks	Change	P-	Baseline	12 Weeks	Change	P-	
IL-1β , pg/ml									
Pre	0.02 (0.20)	0.00 (0.17)	-0.02 (0.34)	***0.382***	0.05 (0.10)	0.00 (0.02)	-0.02 (0.10)	***0.463***	***0.373***
Post	0.06 (0.19)	0.00 (0.13)	0.00 (0.12)	***0.540***	0.06 (0.20)	0.00 (0.03)	-0.03 (0.08)	***0.034***	***0.548***
P-value	***0.344***	***0.721***			***0.801***	***0.715***			
IL-2, pg/ml									
Pre	0.28 (0.80)	0.13 (0.91)	-0.21 (1.31)	***0.470***	0.02 (1.07)	0.00 (2.13)	0.00 (0.44)	***0.779***	***0.648***
Post	0.20 (1.16)	0.39 (0.68)	-0.15 (1.60)	***0.177***	0.14 (1.94)	0.00 (1.98)	0.00 (0.26)	***0.327***	***0.755***
P-value	***0.320***	***0.610***			***0.333***	***0.463***			
IL-6, pg/ml									
Pre	0.89 (1.21)	1.03 (0.93)	0.14 (0.55)	***0.363***	0.72 (1.11)	0.80 (0.85)	0.01 (1.78)	***0.814***	***0.867***
Post	0.77 (1.21)	0.83 (1.20)	0.26 (1.73)	***0.733***	0.82 (1.54)	1.33 (1.04)	0.70 (2.00)	***0.182***	***0.427***
P-value	***0.055***	***0.650***			***0.256***	***0.006***			
IL-7, pg/ml									
Pre	0.00 (0.00)	0.00 (0.05)	0.00 (0.05)	***0.753***	0.04 (0.29)	0.00 (0.31)	-0.04 (0.60)	***0.735***	***0.516***
Post	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	***1.00***	0.01 (0.15)	0.04 (0.65)	0.00 (0.71)	***0.917***	***1.000***
P-value	***0.285***	***1.000***			***0.673***	***0.686***			
IL-8, pg/ml									
Pre	2.91 (2.22)	3.44 (2.47)	-0.29 (3.58)	***0.514***	2.79 (2.62)	3.22 (1.64)	0.67 (3.45)	***0.875***	***0.581***
Post	3.07 (2.57)	3.95 (3.60)	0.72 (6.78)	***0.496***	3.34 (1.57)	3.65 (2.51)	0.52 (3.18)	***0.410***	***0.981***
P-value	***0.868***	***0.733***			***0.255***	***0.012***			
IL-10, pg/ml									
Pre	8.38 (7.26)	10.38 (12.53)	3.03 (15.48)	***0.427***	7.02 (8.58)	10.30	3.17 (9.38)	***0.209***	***1.000***
Post	6.88 (6.82)	10.08 (8.68)	2.21 (11.01)	***0.427***	8.62 (8.81)	7.84 (4.77)	0.10 (10.04)	***0.530***	***0.683***
P-value	***0.266***	***0.570***			***0.836***	***0.071***			

IL-12, pg/ml									
Pre	0.00 (2.14)	0.17 (3.28)	0.00 (2.65)	***0.314***	1.27 (2.39)	1.13 (3.90)	-0.10 (2.88)	***0.594***	***0.516***
Post	0.23 (1.22)	0.00 (1.68)	0.00 (0.72)	***0.563***	0.88 (2.93)	0.82 (4.56)	0.00 (5.61)	***0.208***	***0.277***
P-value	***0.959***	***0.017***			***0.333***	***0.575***			
IFN-γ , pg/ml									
Pre	2.76 (18.20)	5.38 (8.06)	0.00 (13.50)	***0.861***	5.34 (17.56)	8.09	2.10 (30.05)	***0.814***	***0.943***
Post	1.26 (4.63)	1.15 (2.33)	-0.30 (6.86)	***0.463***	3.93 (8.06)	3.06	-1.20 (18.95)	***0.754***	***0.943***
P-value	***0.249***	***0.010***			***0.311***	***0.213***			
GM-CSF, pg/ml									
Pre	0.45 (0.48)	0.31 (0.89)	-0.09 (0.82)	***0.427***	0.69 (1.32)	0.68 (0.75)	-0.26 (0.79)	***0.638***	***0.867***
Post	0.66 (0.73)	0.27 (0.58)	-0.22 (0.71)	***0.022***	0.75 (2.32)	0.69 (0.54)	-0.30 (2.10)	***0.530***	***0.347***
P-value	***0.289***	***0.087***			***0.039***	***0.248***			
TNF-α , pg/ml									
Pre	7.90 (8.32)	7.00 (6.13)	0.27 (11.70)	***0.865***	4.88 (1.78)	3.89 (3.82)	-1.10 (8.65)	***1.000***	***0.792***
Post	6.50 (9.34)	6.69 (4.78)	0.14 (11.72)	***0.691***	4.40 (6.85)	5.42 (8.62)	0.86 (14.03)	***0.583***	***0.581***
P-value	***0.670***	***0.532***			***0.171***	***0.433***			
NE, ng/ml									
Pre	300.85	311.96	47.50	***0.136***	174.53	238.27	102.44 (476.62)	***0.007***	***0.848***
Post	417.15	361.17	-73.58 (99.73)	***0.068***	269.05	376.66	19.72 (511.23)	***0.492***	***0.007***
P-value	***0.035***	***0.691***			***0.031***	***0.279***			

5.3.15 Summary of findings

The actions of each of the above cytokines are described in *Table 9* which has also categorised the cytokines into pro or anti-inflammatory. From this it is clear that prior to the 12 week SET, patients with IC in both groups display increased levels of the pro-inflammatory cytokines IL-1β and TNF-α. In contrast, levels of pro-inflammatory cytokines IL-7 and IL-12 were below levels observed in healthy humans, while the anti-inflammatory cytokine IL-10 was elevated above the expected range.

A similarly mixed picture is seen following the implementation of the exercise intervention. In Group A, homocysteine, a nonspecific marker of inflammation increased over the duration of the SET to a significantly greater level than the change observed in Group B. Also in Group A, the pro-inflammatory cytokine NE increased following an acute bout of exercise at the start of the 12 week program. In contrast, at the end of the SET, the pro-inflammatory cytokines IL-12 and IFN-γ were found to decrease after an acute bout of exercise. Levels of the pro-inflammatory cytokine GM-CSF were significantly less after an acute bout of exercise at the completion of the SET compared with at the start of the SET.

In Group B, the pro-inflammatory NE increased following an acute bout of exercise at the commencement of the SET and pro-inflammatory cytokines IL-6 and IL-8 increased after an acute bout of exercise at the completion of the SET. The relative increase in NE after an acute bout of exercise at the completion of the SET compared with at the start of the SET was significantly greater than the equivalent relative decrease observed for this in Group A. In contrast, levels of the pro-inflammatory IL-1β were significantly less after an acute bout of exercise at the completion of the SET compared with at the start of the SET. Also in Group B, the number of lymphocytes was found to decrease over the duration of the SET.

Table 9: Cytokines analysed in the study presented in this thesis, their normal range and the role they play in the inflammatory response.

Cytokine	Function
Interleukin 1β (range: normally undetectable)(315)	Pro-inflammatory: Activation of leukocytes and the vascular endothelium, stimulate proliferation of fibroblasts, induce systemic acute-phase responses including pyrexia
Interleukin 2 (range: 0-5pg/ml)(316)	Pro-inflammatory: Has a role in growth, proliferation and differentiation of T-cells and can facilitate immunoglobulin production
Interleukin 6 (range: 0.01-11.5pg/ml)(317)	Pro-inflammatory: Responsible for stimulating acute-phase protein synthesis and production of neutrophils. Can also inhibit regulatory T-cells and support growth of B-cells
Interleukin 7 (range: 1-20pg/ml)(318)	Pro-inflammatory: A haematopoietic growth factor associated with survival, proliferation, maturation and differentiation of B and T lymphocytes
Interleukin 8 (range: 0-18pg/ml)(319)	Pro-inflammatory: Associated with neutrophil chemotaxis and degranulation
Interleukin 10 (range: <5.0pg/ml)(320)	Anti-inflammatory: Inhibits activation and effector function of T-cells, monocytes and macrophages. Also regulates growth and differentiation of multiple other cell types.
Interleukin 12 (range: 35-182pg/ml)(321, 322)	Pro-inflammatory: Stimulates the growth and function of T-cells and induces production of interferon-gamma and TNF-α lpha
Interferon-gamma (range: 0-20pg/ml)(323, 324)	Pro-inflammatory: Involved in the regulation of nearly all stages of inflammatory response including activation, growth and differentiation of T-cells, B-cells and macrophages
Granulocyte Macrophage Colony Stimulating Factor (range: <2.0pg/ml)(325)	Pro-inflammatory: Stimulates haematopoietic stem cells to produce granulocytes and monocytes
Tumor Necrosis Factor-alpha (range: detectable in approx. 15% elderly humans)(326)	Pro-inflammatory: Activation of leukocytes and the vascular endothelium, stimulate proliferation of fibroblasts, induce systemic acute-phase responses including pyrexia
Neutrophil Elastase (range: <200pg/ml)(327)	Pro-inflammatory: A protease expressed primarily in the granules of neutrophils that is a potent chemotactic agent. It regulates the activities of numeorus cytokines and cell-surface receptors

5.4 Discussion

Macrophages, T cells, pro-inflammatory cytokines and CAM's have been identified in atherosclerotic lesions supporting the hypothesis that innate and adaptive immune responses participate in the mechanism of atherogenesis (328). Despite this, previous studies have looked at individual or only a handful of markers of inflammation, without undertaking an in depth analysis of the impact of SET on a broad range of cytokines and other markers of immune response.

There are however, a number of authors who have assessed the impact of PAD on the inflammatory and immune response of these patients and an understanding of this may provide a platform with which to interpret the influence of SET on such a response. Referred to as "*Inflammatory profiling of Peripheral Arterial Disease*" by Chaparala et al(329), given the systemic pro-inflammatory state associated with atherosclerosis, it is not surprising that there is evidence demonstrating a chronic elevated baseline level of cytokines and other inflammatory markers in patients with PAD. Signorelli et al (203) found that IL-6 and TNF-α were greater at rest in patients with IC compared with non-PAD controls, while similar findings were reported by Andreozzi et al (202) with respect to IL-1β and IL-6. Interestingly, in contrast to the findings above, Fiotti et al (192) investigated patients with IC, CLI and healthy controls and detected no difference in the baseline levels of IL-1β, IL-6 and TNF-α among the three groups.

In order to provide a broader understanding of the impact of PAD on inflammatory response and immune function, both Chaparala et al (329) and Botti et al (330) used multiplex cytokine analysis to profile an array of pro and anti-inflammatory markers. Botti et al (330) demonstrated raised levels of IFN-γ and TNF-α in the serum of

patients with CLI compared with healthy controls, however, there were no changes observed in levels of interleukins 1a, 1b, 2, 4, 6, 8 and 10. Chaparala et al (329) assessed both patients with IC and CLI compared with healthy controls and reported significantly greater levels of IL-6, IL-10 and IL-13 in patients with PAD. Interleukins 1b, 2, 4 and 8 were not different between the groups.

Although not specifically designed to address the impact of PAD on inflammatory and immune response, the study presented in this thesis also utilised multiplex analysis to profile an array of cytokines. Results of the study presented in this thesis suggest that patients with IC have higher than normal levels of IL-1β, IL-10, NE and TNF-α and unique to the literature, also demonstrate lower than expected levels of pro-inflammatory cytokines IL-7 and IL-12. Unexpectedly high or low baseline levels of interleukins 2, 6 and 8, IFN-γ or GM-CSF were not observed. When considering results of the study presented in this thesis, it is important to recognise that a non-PAD control group was not included and cytokine levels were considered to be "higher" or "lower" than normal on the basis of previously published reference ranges relating to healthy individuals *(See Table 9)*. Of course such reference ranges may vary according to factors such as age, ethnicity, and sensitivity and specificity of a given assay and therefore results should be interpreted with caution.

Despite this obvious limitation in the current study, it is interesting to note the contrasting findings observed throughout the literature. For example, Signorelli et al(203), Andreozzi et al(202) and Chaparala et al(329) all reported raised levels of IL-6 in PAD patients compared to controls, while in the study presented in this thesis, as well as in Fiotti et al(192) and Botti (330), IL-6 was found to be normal. It is likely that once again, the sensitivity and specificity of the various laboratory assays utilised throughout each of these studies would be a contributing factor to such contrasting

results, however, consideration should also be given to the possibility of type 1 or 2 statistical error given the relatively small sample sizes of some of the studies (</= 20 PAD patients in Botti et al (330), Fiotti et al(192) and Signorelli et al(203)).

Furthermore, it is worth mentioning that Botti et al (330) considered only patients with CLI, while the other above mentioned studies including the study presented in this thesis, considered only patients with IC or, as was the case with Fiotti et al (192) and Chaparala et al (329), considered patients with IC or CLI as separate groups. Previous work, including that from Chaparala et al (329), has demonstrated a greater pro-inflammatory burden as the severity of PAD progresses (331). As mentioned above, results from Fiotti et al (192) are in contrast to this and their finding that the receptor antagonist of IL-1β (IL-1ra) increases in correlation with the severity of PAD is suggestive of activation of a compensatory anti-inflammatory mechanism. The implications of such a finding are twofold. Firstly, it supports the concept that the inflammatory profile of patients with IC is different to that of patients with CLI. More importantly however, it provides further insight into the pathophysiological mechanisms associated with atherosclerosis and specifically PAD.

This also brings to light the potential significance of the results observed in the study presented in this thesis, demonstrating higher than expected levels of the anti-inflammatory cytokine IL-10 and lower than expected levels of the pro-inflammatory cytokines IL-7 and IL-12. The higher level of IL-10 supports the previous finding of Chaparala et al(329), while levels of IL-7 and IL-12 have not previously been reported in the literature with respect to PAD patients. Taken together, these results could suggest an adaptive anti-inflammatory process associated with the presence of chronic PAD or a detrimental immunosuppressive phenomenon.

It seems then that atherosclerosis is not simply the systemic pro-inflammatory state that it was once considered to be but rather it precipitates a complex immuno-modulatory state which remains incompletely understood by scientists and physicians alike. Also unclear is whether the pattern of cytokine regulation is different in patients with PAD compared to those with CAD.

Ischaemic preconditioning has been demonstrated to influence cytokine expression after focal brain ischaemia(332). Notably, such an effect was selective, with levels of some cytokines (IL-1β and IL-6) decreased in the pre-conditioned model compared with the non pre-conditioned model, while levels of other cytokines (TNF-α and TGF-β) were not altered by pre-conditioning. The conclusion was therefore made that the impact of preconditioning can lead to different regulation of various cytokines and subsequently different functional properties.

While similar work has not been undertaken in PAD patients, the same model can potentially be applied. The concept of ischaemic preconditioning was first described by Murry et al (333) as the protective effect against myocardial ischaemic injury induced by preceding brief periods of sublethal ischaemia separated by periods of reperfusion. This is analogous of patients with IC, who, on a daily basis, walk to or beyond the onset of pain (relative ischaemia) and then rest until the resolution of pain (reperfusion) before repeating the cycle. It is possible therefore that such ischaemic preconditioning may be responsible, at least in part, for the selective up or down-regulation of baseline cytokine production in patients with PAD observed in this study and others.

The discussion becomes more complicated when consideration is given to the impact of SET on the inflammatory burden of these patients and furthermore, how this can be influenced by different exercise regimens.

Results of this study have demonstrated that although numerous inflammatory markers can be influenced by either a treadmill-based SET or a combination based SET, there is no clear trend to suggest that one exercise regimen has a more pro or anti-inflammatory effect than the other. Rather, it seems that each exercise regimen had an impact on different pro and anti-inflammatory markers, of which, the change in expression was rarely significant between the two groups.

Highlighting the lack of trend observed from the results are the two markers on which the effect of a SET led to a significant change between groups. The non-protein amino-acid homocysteine has been described as pro-inflammatory and pro-atherogenic which at high levels is an independent risk factor for vascular disease. After 12 weeks of SET, homocysteine levels increased significantly in the treadmill-based group to a median level greater than the recommended laboratory reference range (15umol/L), with a magnitude of change that was also significantly greater than that observed in the combination-based group. These findings are in contrast to previous work demonstrating an adaptive lowering of homocysteine levels following long-term exercise training in healthy adults (223) and suggest a detrimental effect of treadmill-based training in patients with IC. This concept however, is not supported by the observed changes in NE levels.

Neutrophil elastase is a serine protease primarily expressed in the granules of neutrophils that is secreted into the extracellular environment following neutrophil activation at sites of inflammation (334). It is a potent chemotactic agent and can

regulate the activities of chemokines, cytokines and cell surface receptors. Given that active stretching of muscles during exercise in healthy individuals is known to lead to recruitment and activation of neutrophils (190) and that neutrophil activation has been implicated in the pathogenesis of IRI (194), it is not surprising that, in both groups, results of the study presented in this thesis demonstrated significantly higher levels of NE following an acute bout of exercise at the commencement of the SET, when compared with pre-exercise levels. Although absolute neutrophil count was not measured acutely post exercise in the study presented in this thesis, results support the findings of Edwards et al(196) who demonstrated a significant increase in absolute neutrophil count following five minutes of treadmill based exercise in patients with IC.

Interestingly, at the completion of the SET, contrary to what the above discussion would suggest, an acute rise in NE was not detected in either group. In the combination based group a significant rise in NE was detected in the resting level of participants from baseline to the completion of the SET which may be reflective of a cumulative pro-inflammatory effect of the SET or an indication that there is a greater proportion of activated neutrophils at rest limiting the capacity of exercise to further increase the NE secretion. Alternatively, as may be the case with the treadmill-based group, the lack of an acute post-exercise increase may represent an adaptive anti-inflammatory response to the repetitive exposure to IRI throughout the SET. In support of this theory, is work from Turton et al (335) who reported that patients with IC developed significant neutrophil activation and degranulation following an acute bout of exercise which decreased sequentially after three months of treadmill training. Furthermore, results from this study demonstrate that the acute post-exercise value of NE in the treadmill based group decreased, from that observed at the start of the SET

to the end of the SET, such that the change in value was significantly less than the corresponding increase in value in the combination training group. Given that the exercise regimens in this study were designed to expose those in the treadmill-based group to a greater frequency of IRI, this finding is consistent with the adaptive anti-inflammatory response proposed above. A possible explanation for such a response may be the intrinsic negative feedback mechanism in which NE is able to inhibit neutrophil stimulation and subsequent NE release by cleavage of immunoglobulins and members of the complement pathway (336).

In light of these findings, it could be expected that changes in neutrophil levels would be observed. As mentioned previously, the study presented in this thesis did not measure neutrophil levels acutely post exercise, however, resting levels of neutrophils were assessed before the SET and at the completion of the SET and no significant change in level was observed in either group. It is possible that while the absolute neutrophil count was the same, there was a change in the proportion of activated neutrophils. Such a concept could be investigated by the flow cytometric assessment of neutrophil CD11b, a marker of neutrophil activation, as was undertaken by Turton et al (335).

When considering markers of systemic inflammatory burden and progression of atherosclerosis, neutrophils are not the only relevant white blood cell subtype. Lymphocytopenia is a physiological response of the innate immune system to systemic inflammation and has been described as cellular immunosuppression that may precipitate the systemic inflammatory response syndrome (337). The fact that participants in the combination based SET were observed to have a significant reduction in lymphocyte levels after the 12 week SET may suggest a detrimental effect of such an exercise regimen.

While regular, moderate intensity exercise has been shown to be protective against infection and potentially malignancy (338), repeated, strenuous exercise may precipitate a state of immunosuppression. A review from Pedersen et al (338) discussing the effect of exercise on lymphocytes and cytokines, suggests that intense, long-term exercise can lead to low concentrations of lymphocytes, suppressed natural immunity and high levels of circulating cytokines. Raised levels of the catecholamines adrenaline and noradrenaline are likely to mediate the acute effect of high intensity exercise on lymphocytes, while increased secretion of the stress hormone cortisol may help to maintain the longer-term lymphocytopenia (338, 339). Other mechanisms include alterations in metabolic function (338).

There is no previous work assessing the impact of SET on lymphocytes in patients with IC. Perhaps the closest analogous study is that from Neves et al(339), who demonstrated that resistance exercise sessions do not provoke acute immunosuppression in older women. Of course, direct comparison with this work is limited by the fact that Neves et al(339) did not consider the impact of treadmill training in combination with resistance training and their work was undertaken in a cohort of healthy older women, who, in the absence of PAD, were not exposed to IRI.

A review article assessing the role of lymphocytes in IRI demonstrated that lymphocytes contribute to the pathogenesis of IRI and mediate tissue injury and possible tissue repair. Suppression of lymphocyte activation has been shown to be beneficial in limiting tissue injury associated with IRI (340).

It is possible then that the reduction in lymphocytes in the combination based training group was an adaptive physiological response to ongoing exposure to IRI. Of course, the study design was such that exposure to IRI was greater in the treadmill-based

group, so if this theory was correct, one would expect to see a greater adaptive response in the treadmill-based group. Instead, the level of lymphocytes was not altered after 12 weeks of SET in this group.

Another possibility is that the relative intensity of the combination based SET in this demographic of patients was sufficient to induce an immunosuppressive rather than immuno-protective effect. Again, if this is the case, it is surprising that a similar observation was not made in the treadmill-based group given that participants in each group used the well validated Borg scale of exercise intensity (264) to similarly rate the respective SET regimens as moderate intensity. Notably, such subjective reporting is not as accurate as observations made objectively and a limitation of the study presented in this thesis was the absence of measured objective physiological parameters such as heart rate and VO_2max which would have enabled accurate determination of exercise intensity in each group.

Alternatively, it could be considered that systemic inflammatory burden increases more following a combination-based SET, thus provoking the reduction in lymphocytes, which as mentioned above is a physiological response of the innate immune system to systemic inflammation. It is hard to combine results of all inflammatory cytokines that have been measured as part of the study presented in this thesis to allow quantification of the overall impact of the two SET regimens on systemic inflammatory burden. There are however, two well established markers of systemic inflammatory burden that have been assessed; NLR and CRP.

Neutrophil-lymphocyte ratio has been extensively reported in the literature as a readily available marker of inflammation which can aid in risk stratification of patients with various manifestations of atherosclerotic disease and other inflammatory

diseases. While there is no established “normal” laboratory range for NLR, a relatively higher NLR is known to be detrimental and may represent the neutrophilia of inflammation or relative lymphopenia of the cortisol induced stress response. In the study presented in this thesis, the reduction in lymphocytes in the combination based-training group translated to a trend towards an increase in NLR in this group and a change between the two groups that approached, but did not reach, statistical significance. Comparison with other studies is not possible because NLR has not previously been reported in this context.

C-reactive protein is an acute-phase reactant that indicates systemic inflammatory activity. Evidence suggests a strong association between elevated CRP levels and atherosclerotic CVD (341). Its use as an independent marker of increased risk of CVD events has also been endorsed by the American Heart Association (193). Previous work has demonstrated that CRP levels are unaffected by an acute bout of exercise in patients with IC (151, 192, 204). Furthermore, 6-months of a treadmill-based SET also led to no change in CRP levels amongst patients with IC in separate work conducted by Tisi et al (229) and Schlager et al (228). Results from the study presented in this thesis demonstrated no change in CRP levels in either exercise group and are consistent with the previously reported findings.

On the basis of results from NLR and CRP, it would appear that SET in patients with IC does not increase systemic inflammatory burden and therefore it is unlikely that such an inflammatory burden is responsible for the observed reduction in lymphocytes in the combination based exercise group. Of course, such markers are non-specific and may not be entirely representative of all physiological inflammatory processes that are occurring. Caution must therefore be exhibited in drawing such a conclusion.

Regardless of the reason, if further work can confirm this potential detrimental immunosuppressive effect of combination-based SET then the likelihood of compounding the well-established concept of age-related immunosenescence cannot be understated. Longer-term follow-up studies would be required to determine whether or not such a finding manifests itself as a greater incidence of infective and/or malignant events in these participants in the future.

The concept of immunosuppression warrants further discussion when consideration is given to the well documented pro-inflammatory response that is expected to occur following an acute bout of exercise (190). In healthy individuals, an acute bout of exercise precipitates increased expression of IL-1β and TNF-α (190). The paracrine actions of theses cytokines stimulates a cascade of molecular activation and additional pro-inflammatory cytokines including IL-6 and IL-8 are also released, ultimately facilitating recruitment of neutrophils. It is proposed that such a transient physiological response provides a means of repair to skeletal muscle damaged during exercise (190).

In the study presented in this thesis, as has been discussed, levels of NE increased acutely following exercise in both groups at the start of the SET. Within both groups, no other measured cytokine demonstrated increased levels following an acute bout of exercise at the commencement of the SET. This lack of an observed acute pro-inflammatory response to exercise is an important finding. Obviously such findings at the first exercise session of 12 weeks of SET cannot lead to the conclusion that SET itself is immunosuppressive, however, in light of the prior discussion highlighting the "abnormal" baseline levels of several cytokines, the current findings lend further support to the concept that the disease state PAD may be somewhat immunosuppressive.

What was observed following an acute bout of exercise at the completion of the SET was not consistent with results at the start of the SET and suggests some impact of SET on immune function. In the combination based exercise group, the pro-inflammatory cytokines IL-6 and IL-8 did increase following acute exercise at the end of the SET. In contrast, in the treadmill-training group, the pro-inflammatory cytokines IL-12 and IFN-γ were observed to decrease following an acute bout of exercise at the completion of the SET. With the exception of NE in the combination based group, the lack of observed change in resting levels of all cytokines, in either group, throughout the duration of the SET suggests no cumulative pro or anti-inflammatory effect of the SET, however, the different changes in cytokine expression following acute exercise at the completion of the SET warrant further exploration.

This discussion has previously outlined the role of ischaemic pre-conditioning and the concept that regular, moderate intensity exercise can enhance the immune system while, repeated, strenuous exercise may precipitate a state of immunosuppression (338). It is possible then that the increased exposure of participants in the treadmill-only exercise group to IRI provided a greater pre-conditioning effect throughout the SET that manifest itself as a beneficial adaptive anti-inflammatory response to an acute bout of exercise. Alternatively, although subjectively exercise intensity was equivalent between the two exercise groups, such increased exposure to IRI and associated oxidative stress may be perceived by the body as higher intensity exercise than that in the combination-based training group, with a resultant immunosuppressive effect on cytokine production.

The pro-inflammatory response observed in the combination-based group could be perceived as a “normal” response to an acute bout of exercise that was absent at the

commencement of the SET. This may suggest that such an exercise regimen has the capacity to provide beneficial immunomodulation or it may be representative of the changes in skeletal muscle mass as a result of such an exercise regimen. As previously mentioned, it is proposed that an acute pro-inflammatory response to exercise provides a means to repair skeletal muscle damaged during exercise (190). Furthermore, skeletal muscle continually produces cytokines in an effort to maintain homeostasis and regulate function (190). Therefore, the gain in skeletal muscle mass observed during the SET in participants undergoing combination based training (*See Chapter 6)* may be responsible for the changes in cytokine expression observed in this group following an acute bout of exercise. Similarly, the relative loss of muscle mass in the treadmill-only group (*See Chapter 6)* may be due to an immunosuppressed state and inability to produce the necessary cytokines to repair muscle fibres injured during exercise.

The measurement of this acute inflammatory response to exercise in patients with IC has previously been undertaken in the literature independent to SET (151, 202, 203), while assessment of such an acute response at the completion of 12 weeks of SET has previously been reported by Turton et al (335) who demonstrated reduced levels of neutrophil activation and degranulation at the completion of 12 weeks of SET in 46 patients with IC. The assessment undertaken in the study presented in this thesis allowed for determination of the impact of SET on the resting inflammatory burden of patients with IC, but also its impact on the inflammatory response to an acute bout of exercise. Unlike Turton et al (335), the marker of neutrophil activation and degranulation (NE) in the study presented in this thesis was found not to decrease after 12 weeks of SET.

Also unique to the study presented in this thesis is the fact that nowhere in the literature has the impact of combination based SET been assessed with respect to its impact on inflammation. Several studies have assessed the impact of treadmill-based training, following 12 weeks of SET, on inflammatory markers (228, 229, 342).

In studies measuring markers of inflammation after an acute bout of treadmill-based exercise in patients with IC, without subjecting participants to a SET (equivalent to an acute bout of exercise at the start of the SET in the current study), in contrast to the results of the study presented in this thesis, Signorelli et al (203) and Andreozzi et al (202) demonstrated a significant increase in inflammatory cytokines (IL-6, IL-1β and TNF-α) in blood samples taken immediately following the completion of exercise. Another study, Palmer-Kazen et al(151), measured IL-6 levels 2, 15, 60 and 120 minutes after exercise. A significant increase in IL-6 was demonstrated only after 120 minutes of recovery from exercise. The absence of a change in IL-6 immediately following exercise supports the results of the study presented in this thesis. It is possible that a late increase in IL-6 would also have been observed in the current study if blood samples had been taken at similar time points. The reason for conflicting results amongst these studies may be due to the fact that unlike the study presented in this thesis in which acute exercise was considered to be a one-hour supervised session, these studies subjected patients with IC to a single treadmill test only and did not quantify intensity.

Furthermore, only the study presented in this thesis considered a panel of cytokines following acute exercise. The consistency of results across this panel, with the exception of NE which was discussed previously, adds strength to the results of the study presented in this thesis compared with others.

Of those studies to have assessed the impact of SET on inflammation, both Tisi et al (229) and Schlager et al (228) measured CRP and fibrinogen as markers of systemic inflammatory burden, as well as the cytokine IL-6 and demonstrated no increase in values following 6 and 12 months of SET respectively. Tisi et al (229) also measured SAA as a marker of inflammation and noted a reduction in level after 6 months of SET. Consistent with the findings of these studies, CRP was measured in the study presented in this thesis and found not to be influenced by SET. The study presented in this thesis did not however, measure fibrinogen and SAA so direct comparison is not possible. In light of these findings and given that homocysteine (a marker of inflammatory burden) increased in the study presented in this thesis after treadmill based-exercise, it seems that SET in patients with IC may selectively impact on markers of systemic inflammation.

In another study, Nowak et al (342) subjected participants to a 12 week treadmill-based SET comparable to the regimen used in the study presented in this thesis and measured the inflammatory response using a multiplex cytokine panel also analogous to that used in the study presented in this thesis. The only significant change observed was a reduction in resting IL-6 levels at the completion of the SET. Like the study presented in this thesis, expression of all other cytokines was unchanged. Although the current study did not demonstrate a decreased level of IL-6 post SET, consistent with Schlager et al (228), this finding in combination with the reduced levels of pro-inflammatory cytokines IL-12 and IFN-γ after an acute bout of exercise at the end of the SET in the study presented in this thesis add weight to the concept of an adaptive anti-inflammatory or immunosuppressive impact of treadmill-based SET.

To summarise, exercise in healthy individuals can demonstrate an acute pro-inflammatory response with adaptive anti-inflammatory or immunosuppressive

changes depending on duration and intensity of exercise. In PAD, it seems that the disease process itself creates a complex immune-modulatory state and the impact of exercise is difficult to quantify. Findings of the study presented in this thesis raise the possibility that exercise in patients with IC and indeed SET, may induce an acute pro-inflammatory response, a beneficial adaptive anti-inflammatory response or a detrimental immunosuppressive response. Furthermore, it is difficult to say whether a treadmill-based training or combination-based training regimen is more beneficial than the other until it is possible to quantify whether the observed changes are real or a result of statistical error and whether or not the changes are adaptive anti-inflammatory or immunosuppressive.

The concept of statistical error is important given that assessment of inflammatory markers is a secondary outcome of the study presented in this thesis and the inability to detect differences in many cytokine levels might be due to an underpowered population. Findings should therefore be interpreted with caution until larger scale studies are undertaken. These should not only assess an array of inflammatory markers, as was the case with the study presented in this thesis, but also attempt to quantify a potential immunosuppressed state through the measurement of monocyte surface antigen expression as well as reporting clinical outcomes, in particular the incidence of infectious events of those patients with IC undertaking a SET compared to those who are not.

CHAPTER 6: RESULTS – IMPACT OF THE INTERVENTION ON BODY COMPOSITION, ENERGY EXPENDITURE, DIETARY INTAKE AND PROTEIN EXPRESSION

6.1 Introduction

The nutritional vulnerability of patients with PAD has been recognised and exercise is known to increase metabolic demands, potentially leading to a decline in nutritional status and physical health in this vulnerable group of patients (248). Despite this and the fact that evidence exists to suggest that combined nutrition and exercise interventions in the rehabilitation setting are preferable (259, 260), there is a scarcity of work investigating the impact of SET on dietary intake, body composition and nutritional status. Preservation of SMM is particularly important in achieving optimal outcomes from exercise interventions (252). Resistance training is known to improve SMM (343) and gains in SMM are likely to translate into better functional outcomes and walking performance (248). Skeletal muscle mass can be influenced by dietary intake, in particular protein, and energy expenditure (252). Furthermore, the contribution of SMM to whole body protein metabolism is also an important determinant of response to physiological and pathological stress and its impact on REE may be protective against the onset of obesity (252). Quantitative assessment of SMM and body composition as well as REE can be achieved through the use of DEXA and indirect calorimetry respectively.

At a molecular level, the calcium dependent cysteine protease calpain system and its endogenous inhibitor calpastatin may be activated by changes in intra-cellular calcium concentration associated with exercise leading to protein turnover by way of apoptosis and myogenesis (171). Ischaemia-reperfusion injury is known to further raise

intracellular calcium levels, leading to increased calpain activation, increased protein turnover and possibly skeletal muscle wasting (344). High levels of calpain activation have also been implicated with a failure of angiogenesis. The ability of the calpain system to adapt to variations in intracellular calcium levels, thus allowing angiogenesis and preventing excessive skeletal muscle wasting, may be a key factor in determining response to exercise in these patients.

6.2 Aim

The aim of this chapter is to address **RQ4:** "Will 12 weeks of SET consisting of interval treadmill and lower limb resistance training produce more significant gains in skeletal muscle mass compared to an exercise regimen consisting of treadmill training alone in patients with IC and if so, can this be explained by changes in calpain and calpastatin activity, dietary intake or resting energy expenditure?"

6.3 Results

Table 10 highlights the change in the outcome measures relevant to RQ4 from baseline to 12 weeks according to allocation, for both ITT and per-protocol analyses. There were no statistically significant differences observed between groups at baseline.

6.3.1 Total body weight

After 12 weeks of SET, there was no change in total body weight in either Group A or Group B (Table 10).

6.3.2 Body Composition

Access to the DEXA facility was unavailable for the first six participants while a further two participants declined involvement in this component of the study. Both of these participants cited the time burden that was already associated with other

requirements of the study as their reason for this. Data were therefore collected from 14 (9M, 5F) and 13 (10M, 3F) participants in Group A and B respectively. Of these, per-protocol analysis was possible on 11 (8M, 3F) participants in Group A and 11 (8M, 3F) participants in Group B.

Skeletal muscle mass and FM demonstrated a non-significant decrease between baseline and 12 weeks for those allocated to Group A. A non-significant increase in SMM and FM was observed between baseline and 12 weeks for those allocated to Group B. On per-protocol analysis statistical significance was achieved for between group differences with respect to SMM but not FM.

Of the 14 participants in Group A and 13 participants in Group B who underwent DEXA scans, 13 participants (Group A=5, Group B=8) experienced unilateral IC and the remaining 14 (Group A=9, Group B=5) experienced bilateral IC. Considering each leg as a separate entity, there were therefore 23 symptomatic legs in Group A and 18 symptomatic legs in Group B.

When data obtained from DEXA scanning were focused to assess only SMM and FM of these symptomatic legs, SMM decreased non-significantly for those allocated to Group A. In contrast, SMM of the symptomatic leg for those allocated to Group B increased in both ITT and per-protocol analyses and while this within group change from baseline to 12 weeks did not reach statistical significance on ITT analysis, it was statistically significant on per-protocol analysis (P=0.04). These results translated into a borderline statistically significant difference between groups on ITT analysis (P=0.05) and clear significance on per-protocol analysis (P=0.02).

Small non-significant gains in FM of symptomatic legs were observed in both groups with ITT and per-protocol analysis.

A focused assessment was also undertaken of the SMM and FM in the asymptomatic legs of the 13 participants who suffered from unilateral IC only (Group A=5, Group B=8). There was an increase in SMM from baseline to 12 weeks within groups which achieved statistical significance on per-protocol analysis (Group A=4, Group B=7); P=0.03 and P=0.05 for Group A and Group B respectively. There was no between group difference detected in either ITT or per-protocol analyses.

FM increased in Group A and B. With ITT analysis, this increase was significant in Group B only with no significant difference detected between the groups. Per-protocol analysis showed the increase in FM to be significant in both groups, however the magnitude of increase in Group A was found to be significantly greater than that in Group B.

6.3.3 Calpain System

One participant in each group declined to consent for a muscle biopsy, citing the invasive nature of the procedure as the reason. Baseline muscle biopsies were therefore performed on 17 participants in Group A and 16 participants in Group B. A further patient in Group A declined a post-intervention biopsy as he found his baseline biopsy to be an unpleasant experience. Participants who were forced to withdraw from the program were not asked to undergo repeat biopsies, however, for ITT analysis, data from their baseline biopsy was carried forward. Per-protocol analysis was therefore undertaken on 14 participants in Group A and 12 participants in Group B.

Calpain activity increased in Group A and decreased in Group B between baseline and 12 weeks for ITT analyses, reaching statistical significance within Group A only. The between group comparison approached statistical significance and when per-

protocol analyses were performed, the within and between group differences were similar in magnitude and statistical significance. Calpastatin activity did not achieve statistical significance within or between groups.

The change in calpain activity was found to correlate poorly with changes in SMM in Group A (r=-0.20, p=0.56) and Group B (r=-0.23, p=0.48). No difference was observed between baseline calpain activity in gastrocnemius muscle compared to that of vastus medialis muscle in Group A [1.62×10^5 (9.47×10^4) versus 1.80×10^5 (1.25×10^5), p=0.63] or Group B [1.74×10^5 (9.02×10^4) versus 1.73×10^5 (8.70×10^4), p=0.99.]

6.3.4 Resting Energy Expenditure (REE)

Indirect calorimetry was available for a subset of participants due to difficulty with access to the equipment. The procedure was therefore undertaken both at baseline and post-intervention on 17 patients (Group A=9, Group B=8) who were analysed for ITT purposes. Of these participants two did not complete the intervention meaning per-protocol analysis involved eight participants in Group A and seven participants in Group B.

Table 10 shows REE results expressed in kilojoules and then adjusted for both total body weight (kj/kg) and total SMM (kj/kg_SMM). In each case, the exercise intervention undertaken in both Group A and Group B did not significantly impact on REE both unadjusted and adjusted. Furthermore, no significant difference was observed in REE between groups.

6.3.5 Dietary Intake

Data on dietary intake were collected from all patients who submitted to undertake DEXA analysis. Data were therefore collected from 14 (9M, 5F) and 13 (10M, 3F)

participants in Group A and B respectively. Of these, per-protocol analysis was possible on 11 (8M, 3F) participants in Group A and 11 (8M, 3F) participants in Group B.

Table 11 shows the acceptable macronutrient distribution range (AMDR) and SDT's for selected nutrients.

The median (IQR) macronutrient and micronutrient intake of participants in Group A and Group B both before and after 12 weeks of SET is presented in *Table 12*. At both time points, while the majority of participants in each group achieved the AMDR for protein, there were few participants achieving the AMDR for carbohydrate, total fat, saturated fat, polyunsaturated fat and none achieved the AMDR for monounsaturated fat. Similarly, there were few participants in either group who achieved SDT's for fibre, cholesterol, folate, potassium, sodium, retinol equivalents, vitamin C and none achieved the SDT for vitamin E.

For each nutrient, the change in dietary intake was not statistically significant either within or between groups over the duration of the SET.

6.3.6 Summary

These results have demonstrated that the impact of SET on body composition in patients with IC is dependent on modality of exercise and may have a different effect on symptomatic compared with asymptomatic legs. Treadmill only training resulted in a loss of SMM in symptomatic legs that was significantly different to the gain in SMM observed in symptomatic legs after combination training. These findings were also reflected in total body SMM. In contrast, gains in SMM were observed in asymptomatic legs irrespective of exercise modality. Fat mass seemed to be influenced to a lesser extent by exercise. Although widespread increases in FM were

noted in both groups, these were generally not significant with the exception of asymptomatic legs in which the increase in FM was found to be significantly greater after treadmill based training compared to combination training.

Calpain activity increased in those undertaking treadmill training and tended to decrease after combination training. Despite this, similar trends were not exhibited in calpastatin activity, the endogenous inhibitor of calpain, which was not significantly impacted on by either exercise regimen.

Resting energy expenditure was associated with large variability and was not altered by exercise in either group, even after adjustment for changes in body composition. Similarly, a 12 week exercise intervention did not lead to changes in the dietary intake of patients with IC which was found to be poor when compared to the recommendations provided by international guidelines.

Table 10: Comparison of baseline, 12 week and absolute change for outcomes between participants in the treadmill only supervised exercise training and the treadmill and resistance exercise training. Data are presented as mean (SD) unless otherwise stated: Body composition, Calpain system, Resting energy expenditure.

Outcome	**Treadmill only**				**Treadmill and Resistance Training**				**Between groups P-*value***
	Baseline	12 Weeks	Change	P-*value*	Baseline	12 Weeks	Change	P-*value*	
Intention to treat									
Total body weight, kg	77.3 (19.6)	77.0 (18.7)	-0.3 (1.5)	0.51	81.0 (17.3)	81.9 (17.2)	0.9 (1.8)	0.11	0.09
Calpain activity, FU ($x10^5$)	1.62 (0.95)	2.21 (1.26)	0.59 (1.24)	0.05	1.67 (0.90)	1.45 (1.39)	-0.22 (1.43)	0.55	0.09
Calpastatin activity, FU ($x10^6$)	2.74 (1.05)	3.13 (1.11)	0.39 (1.45)	0.27	2.93 (1.00)	2.68 (0.92)	-0.25 (1.39)	0.50	0.21
Skeletal Muscle Mass, kg	23.72 (6.77)	23.47 (6.62)	-0.25 (0.55)	0.11	25.43 (5.42)	25.64 (5.52)	0.21 (0.81)	0.38	0.10
Symptomatic leg SMM, kg	7.90 (2.16)	7.80 (2.10)	-0.10 (0.28)	0.10	8.38 (1.65)	8.52 (1.80)	0.14 (0.47)	0.19	0.05

Asymptomatic leg SMM, kg	7.62 (1.65)	7.81 (1.92)	0.19 (0.28)	0.21	7.86 (1.40)	7.96 (1.31)	0.10 (0.20)	0.19	0.58
Fat mass, kg	25.43 (9.79)	25.34 (9.50)	-0.09 (1.13)	0.77	25.64 (8.90)	25.96 (8.85)	0.32 (0.95)	0.26	0.32
Symptomatic leg FM, kg	3.46 (1.24)	3.57 (1.14)	0.12 (0.51)	0.29	3.25 (0.95)	3.30 (0.99)	0.05 (0.20)	0.31	0.57
Asymptomatic leg FM, kg	4.05 (1.26)	4.22 (1.23)	0.17 (0.20)	0.14	2.95 (0.80)	3.10 (0.87)	0.15 (0.20)	0.04	0.93
REE, kj	5614 (1266)	5316 (1216)	-297 (776)	0.28	5819 (1216)	5823 (730)	5 (1078)	0.99	0.52
REE, kj/kg	72.6 (11.7)	68.9 (10.1)	-3.7 (11.6)	0.37	74.9 (9.9)	75.3 (15.8)	0.4 (13.5)	0.95	0.52
REE, kj/kg_SMM	242.6 (34.8)	233.1 (35.0)	-9.5 (40.5)	0.50	241.6 (43.1)	242.0 (44.1)	0.4 (40.7)	0.98	0.62
Per protocol									
Total body weight, kg	80.3 (21.0)	79.9 (20.0)	-0.4 (1.7)	0.51	81.0 (19.0)	81.9 (18.9)	0.9 (2.0)	0.13	0.11
Calpain activity, FU ($x10^5$)	1.35 (0.59)	2.11 (1.25)	0.76 (1.37)	0.05	1.60 (0.99)	1.45 (1.50)	-0.15 (1.55)	0.75	0.13
Calpastatin activity, FU ($x10^6$)	2.64 (1.10)	3.18 (1.19)	0.54 (1.69)	0.28	2.83 (1.02)	2.62 (0.95)	-0.21 (1.53)	0.65	0.27

Skeletal Muscle Mass, kg	22.84 (6.93)	22.64 (6.74)	-0.20 (0.59)	0.28	25.24 (5.91)	25.64 (6.05)	0.40 (0.70)	0.09	0.04
Symptomatic leg SMM, kg	7.96 (2.29)	7.88 (2.21)	-0.08 (0.28)	0.27	8.35 (1.81)	8.59 (1.97)	0.24 (0.40)	0.04	0.02
Asymptomatic leg SMM, kg	8.10 (1.48)	8.40 (1.64)	0.30 (0.16)	0.03	7.70 (1.44)	7.85 (1.38)	0.15 (0.16)	0.05	0.19
Fat mass, kg	26.14 (9.14)	25.92 (9.78)	-0.22 (1.10)	0.51	25.82 (9.71)	25.94 (9.69)	0.12 (0.85)	0.65	0.42
Symptomatic leg FM, kg	3.51 (1.15)	3.55 (1.23)	0.04 (0.25)	0.53	3.25 (1.04)	3.27 (1.09)	0.02 (0.20)	0.76	0.78
Asymptomatic leg FM, kg	3.80 (1.30)	4.10 (1.35)	0.25 (0.07)	0.005	2.93 (0.86)	3.04 (0.92)	0.11 (0.11)	0.04	0.02
REE, kj	5600 (1353)	5365 (1290)	-234 (804)	0.44	5941 (1259)	5797 (784)	-144 (1071)	0.73	0.86
REE, kj/kg	72.2 (11.7)	69.3 (10.7)	-2.9 (12.1)	0.53	76.9 (8.9)	75.4 (17.0)	-1.5 (13.5)	0.79	0.84
REE, kj/kg_SMM	244.7 (36.6)	237.3 (35.0)	-7.4 (42.8)	0.64	250.0 (38.6)	243.5 (47.4)	-6.6 (34.4)	0.67	0.97

Table 11: Acceptable macronutrient distribution range and suggested dietary targets for selected nutrients (345).

Nutrient	Acceptable Macronutrient Distribution Range
Protein	15-25% Total Energy
Carbohydrate	45- 65% Total Energy
Total fat	20-35% Total Energy
Saturated fat	<10% Total Energy
Monounsaturated fat	>20% Total Energy
Polyunsaturated fat	8-10% Total Energy
	Suggested Dietary Target
Cholesterol, mg/day	200
Sodium, mg/day	1600
Potassium, mg/day	4700
Folate, µg/day	300
Fibre, g/day	Male: 38; Female:28
Retinol equivalents, µg/day	Male: 1500; Female: 1220
Vitamin E, mg	Male: 19; Female: 14
Vitamin C, mg	Male: 220 ; Female: 190

Table 12: Dietary intake of participants with IC pre and post supervised exercise training for group 1 and group 2. Data presented as median (IQR) unless otherwise stated.

	Treadmill only				Treadmill and Resistance Training				Between groups P-*value*
	Baseline	**12 weeks**	**Change**	**P-*value***	**Baseline**	**12 weeks**	**Change**	**P-*value***	
Intention to treat	**(n=14)**	**(n=14)**	**(n=14)**		**(n=13)**	**(n=13)**	**(n=13)**		
Protein									
% kJ from protein	20.1	20.2	0.3	0.55	20.5	22.1	0.8	0.46	0.76
	(16.5, 22.4)	(17.7, 22.5)	(-1.4, 1.6)		(17.1, 23.5)	(18.1, 24.4)	(-1.7, 2.9)		
N (%) achieving AMDR	10 (71)	11 (79)			11 (85)	13 (100)			
Carbohydrate									
% kJ from carbohydrate	39.7	41.1	0.1	0.70	41.3	40.0	-1.0	0.42	0.83
	(37.6, 44.5)	(37.1, 44.0)	(-4.1, 2.6)		(37.7, 44.6)	(37.1, 42.4)	(-4.2, 3.2)		
N (%) achieving AMDR	2 (14)	3 (21)			3 (23)	1 (8)			
Total fat									
% kJ from total fat	37.3	36.5	0.4	0.86	34.5	36.8	-0.1	0.75	0.87
	(33.9, 40.4)	(32.9, 40.0)	(-1.7, 2.5)		(33.0, 38.7)	(34.3, 39.5)	(-1.0, 2.8)		
N (%) achieving AMDR	7 (50)	7 (50)			8 (62)	6 (46)			

Saturated fat									
% kJ from saturated fat	14.4	13.8	0.1	0.86	12.2	12.1	0.1	0.80	0.91
	(12.4, 15.9)	(13.3, 16.8)	(-1.1, 1.6)		(10.9, 12.9)	(11.1, 13.3)	(-1.1, 0.6)		
N (%) achieving AMDR	1 (7)	2 (14)			3 (23)	2 (15)			
Monounsaturated fat									
% kJ from monounsaturated fat	13.5	12.6	0.0	0.92	12.6	13.1	0.2	0.19	0.33
	(12.3, 15.9)	(11.0, 13.9)	(-0.6, 0.8)		(11.5, 13.2)	(12.6, 15.6)	(-0.4, 2.2)		
N (%) achieving AMDR	0 (0)	0 (0)			0 (0)	0 (0)			
Polyunsaturated fat									
% kJ from polyunsaturated fat	5.6	6.2	0.2	0.51	7.3	6.6	0.1	0.65	0.87
	(4.3, 6.9)	(4.2, 7.2)	(-0.6, 1.2)		(5.0, 8.7)	(5.9, 9.2)	(-0.4, 1.0)		
N (%) achieving AMDR	1 (7)	2 (14)			2 (15)	2 (15)			
Cholesterol									
mg/day	228.9	257.1	-2.8	0.98	266.2	303.7	-6.6	0.97	0.94
	(178.7, 444.0)	(144.2, 450.2)	(-67.8, 65.0)		(215.3, 349.6)	(197.6, 329.9)	(-83.8, 97.4)		
N (%) achieving SDT	6 (43)	6 (43)			2 (15)	3 (23)			
Sodium									
mg/day	2313.3	2158.0	-99.8	0.42	2405.6	2346.0	-288.3	0.86	0.94
	(1690.2, 2964.2)	(1326.4, 3960.7)	(-690.1, 230.1)		(2107.5, 2793.9)	(1516.5, 3053.5)	(-910.2, 755.5)		

	N (%) achieving SDT	3 (21)	4 (29)			1 (8)	3 (23)			
Potassium										
	mg/day	2871.8	3132.3	15.7	0.97	2794.1	2590.0	363.3	0.86	0.91
		(2052.7, 3829.0)	(1512.8, 4266.6)	(-634.1, 794.1)		(2292.8, 3485.3)	(2260.6, 3033.7)	(-1069.1, 512.3)		
	N (%) achieving SDT	1 (7)	0 (0)			0 (0)	1 (8)			
Folate,										
	µg/day	270.4	292.1	-14.0	0.51	284.2	264.1	4.1	0.92	0.76
		(182.3, 343.4)	(163.6, 375.7)	(-88.6, 58.4)		(205.8, 319.5)	(212.1, 305.6)	(-40.6, 31.5)		
	N (%) achieving SDT	5 (36)	7 (50)			4 (31)	3 (23)			
Fibre										
	g/day	24.6	21.7	0.2	0.65	21.4	22.6	-1.6	0.81	0.72
		(16.3, 25.5)	(14.4, 33.0)	(-4.6, 6.3)		(19.3, 26.2)	(19.0, 27.9)	(-3.0, 3.8)		
	N (%) achieving SDT	0 (0)	0 (0)			2 (15)	1 (8)			
Retinol equivalents										
	µg/day	820.3	755.4	-117.2	0.09	887.0	733.5	-30.9	0.60	0.55
		(614.8, 1098.4)	(444.4, 1099.0)	(-234.8, 7.9)		(658.3, 946.2)	(564.2, 1042.4)	(-210.3, 265.4)		
	N (%) achieving SDT	1 (7)	0 (0)			0 (0)	1 (8)			
Vitamin E										
	mg/day	6.6	4.8	-0.5	0.31	5.8	7.1	0.3	0.42	0.30

	(3.8, 9.3)	(3.9, 10.2)	(-1.4, 0.31)		(5.2, 8.9)	(4.9, 9.9)	(-1.0, 1.1)		
N (%) achieving SDT	0 (0)	0 (0)			0 (0)	0 (0)			
Vitamin C									
mg/day	111.6	119.5	-7.1	0.65	93.3	108.7	1.3	0.55	0.58
	(79.5, 205.4)	(68.6, 229.3)	(-27.9, 38.9)		(80.9, 126.3)	(85.8, 126.8)	(-19.2, 26.3)		
N (%) achieving SDT	3 (21)	4 (29)			0 (0)	0 (0)			
Per Protocol	**(n=11)**	**(n=11)**	**(n=11)**		**(n=11)**	**(n=11)**	**(n=11)**		
Protein									
% kJ from protein	20.9	20.3	0.4	0.53	20.5	21.3	-0.2	1.00	0.72
	(16.5, 23.5)	(16.6, 24.5)	(-1.4, 1.7)		(17.7, 23.6)	(17.3, 24.3)	(-2.0, 2.1)		
N (%) achieving AMDR	7 (63)	9 (82)			9 (82)	11 (100)			
Carbohydrate									
% kJ from carbohydrate	39.5	41.0	0.2	0.86	41.3	40.6	-0.5	1.00	0.82
	(37.0, 43.6)	(36.8, 43.5)	(-3.6, 3.2)		(35.6, 43.8)	(39.3, 43.4)	(-2.5, 3.6)		
N (%) achieving AMDR	1 (9)	2 (18)			2 (18)	1 (9)			
Total fat									
% kJ from total fat	39.0	36.7	0.8	1.00	34.5	36.8	-0.2	0.72	0.92
	(34.6, 40.6)	(32.9, 41.2)	(-3.0, 2.1)		(33.0, 40.1)	(34.2, 39.2)	(-1.1, 1.2)		

N (%) achieving AMDR	6 (54)	6 (54)			6 (55)	5 (45)			
Saturated fat									
% kJ from saturated fat	15.0	13.9	0.2	0.59	12.2	12.1	0.1	0.48	0.45
	(12.4, 16.3)	(13.5, 16.9)	(-1.3, 2.0)		(11.0, 13.0)	(10.6, 13.2)	(-1.2, 0.4)		
N (%) achieving AMDR	0 (0)	1 (9)			1 (9)	2 (18)			
Monounsaturated fat									
% kJ from monounsaturated fat	13.5	12.6	0	0.86	12.9	13.1	0.1	0.48	0.53
	(12.4, 14.5)	(11.1, 14.7)	(-0.8, 0.7)		(12.3, 13.4)	(12.6, 15.6)	(-0.8, 1.6)		
N (%) achieving AMDR	0 (0)	0 (0)			0 (0)	0 (0)			
Polyunsaturated fat									
% kJ from polyunsaturated fat	5.4	6.2	0.2	0.59	6.0	6.4	0.2	0.18	0.77
	(4.2, 6.5)	(3.6, 6.7)	(-0.5, 1.0)		(4.8, 8.7)	(5.9, 10.1)	(-0.3, 1.1)		
N (%) achieving AMDR	1 (9)	1 (9)			1 (9)	2 (18)			
Cholesterol									
mg/day	222.9	279.3	20.3	0.37	285.1	288.3	-24.6	0.33	0.18
	(162.2, 442.9)	(148.0, 540.0)	(-54.3, 90.6)		(257.6, 362.5)	(193.6, 329.8)	(-104.6, 46.1)		
N (%) achieving SDT	5 (45)	5 (45)			1 (9)	3 (27)			
Sodium									
mg/day	2049.5 (1360.7,	1739.0 (1285.2,	-61.0	0.93	2467.7	2346.0	-290.2	0.48	0.34

		2837.5)	3854.7)	(-412.5, 510.3)		(2304.7, 2930.0)	(1660.4, 3019.6)	(-914.3, 429.6)		
	N (%) achieving SDT	3 (27)	3 (27)			0 (0)	0 (0)			
Potassium										
	mg/day	2652.3	2802.9	137.8	0.33	2952.2	2590.0	-362.2	0.33	0.22
		(1840.4, 3480.1)	(1512.0, 4146.2)	(-363.2, 809.3)		(2328.8, 3609.3)	(2314.0, 2943.6)	(-1208.0, 467.8)		
	N (%) achieving SDT	0 (0)	0 (0)			0 (0)	0 (0)			
Folate										
	µg/day	249.3	265.9	17.8	0.86	290.7	264.1	-4.6	0.53	0.53
		(173.2, 327.0)	(157.4, 367.4)	(-62.0, 69.0)		(230.7, 330.7)	(234.9, 297.3)	(-50.2, 28.4)		
	N (%) achieving SDT	3 (27)	5 (45)			4 (36)	2 (18)			
Fibre										
	g/day	21.6	18.1	0.3	0.42	22.2	22.6	-2.5	0.48	0.31
		(15.4, 25.0)	(12.0, 32.5)	(-4.0, 8.0)		(20.2, 27.9)	(19.5, 26.7)	(-3.3, 3.1)		
	N (%) achieving SDT	0 (0)	0 (0)			2 (18)	1 (9)			
Retinol equivalents										
	µg/day	779.0	622.5	-77.8	0.21	909.5	733.5	-76.3	0.42	0.97
		(567.1, 1052.6)	(408.9, 1150.1)	(-232.8, 22.3)		(745.7, 960.3)	(587.0, 925.3)	(-222.9, 261.3)		
	N (%) achieving SDT	1 (9)	0 (0)			0 (0)	1 (9)			
Vitamin E										

	mg/day	6.0	4.3	-0.5	0.657	5.8	7.1	0.2	0.859	0.82
		(3.5, 9.2)	(3.4, 11.1)	(-1.3, 1.0)		(5.3, 9.1)	(5.4, 9.8)	(-1.8, 0.9)		
	N (%) achieving SDT	0 (0)	0 (0)			0 (0)	0 (0)			
Vitamin C										
	mg/day	150.5	112.9	-9.1	0.374	103.1	108.7	-4.6	1.000	0.67
		(67.2, 256.9)	(52.8, 233.6)	(-42.1, 38.5)		(92.4, 128.3)	(87.8, 124.0)	(-23.3, 22.0)		
	N (%) achieving SDT	3 (27)	4 (36)			0 (0)	0 (0)			

6.4 Discussion

The impact of exercise training on body composition and REE, as well as the role of the calpain system and dietary intake in augmenting such an impact, has not previously been investigated in patients with IC. The findings of the study presented in this thesis are therefore unique and unanticipated.

6.4.1 Skeletal muscle mass and the calpain system

The results observed with respect to SMM demonstrate a superiority of combination based SET over a treadmill based program. It seems that the impact of SET on body composition in patients with IC is dependent on modality of exercise and may have a different effect on symptomatic compared with asymptomatic legs. Treadmill only training resulted in a loss of SMM in symptomatic legs that was significantly different to the gain in SMM observed in symptomatic legs after combination training. These findings were also reflected in total body SMM. In contrast, gains in SMM were observed in asymptomatic legs irrespective of exercise modality.

A mechanistic explanation for these changes in SMM may lie with the calpain system. The proteolytic activity of the calpain system is implicated in muscle apoptosis (346) and a significant increase in calpain activity, as was observed in the treadmill based SET group, is therefore likely to cause muscle wasting and may provide a basis for the loss of SMM observed in this group. As has been mentioned, the calpain system is calcium dependent and becomes activated when calcium levels are physiologically increased by stimuli such as IRI (344). This observed increase in calpain activity is therefore felt likely to be precipitated by a greater exposure to IRI induced by repetitive bouts of treadmill exercise. Adding weight to this theory and highlighting the clinical significance of the calpain system, ischaemic cardiac events have linked

high levels of calpain with contractile dysfunction (347), while calpain activity is increased following ischaemic cerebral events (348).

In the combination training group, lack of significant effect on calpain activity is likely due to an interplay of factors counteracting each other. Resistance training has been shown to up-regulate expression of calpain and to an even greater extent its endogenous inhibitor calpastatin (349). Although not previously demonstrated, a potential decrease in actual calpain proteolytic activity may ensue. Furthermore, the discussion above would suggest that exposure to a degree of IRI induced by the treadmill based component of the SET is likely to increase calpain activity. The net effect of such a program is a non-significant decrease in calpain activity. In this way, other mechanisms responsible for gains in SMM associated with resistance training and the protective effect of aerobic training against age-related reductions in SMM, were allowed to proceed without conflict from the calpain system and may explain the beneficial gain in SMM observed in this group of participants. Support for such a concept is provided by the significant gain in SMM that was observed in the asymptomatic legs of participants within both groups. Although many of these limbs demonstrated radiological evidence of atherosclerotic disease, it seems that the presence of clinically significant IRI manifesting as claudication pain and potentially driving activation of the calpain system is the most important precipitant of a loss of SMM arising from a treadmill based exercise intervention in patients with IC.

It could be considered that activity of calpastatin, would change at a rate inversely proportional to that of calpain and may be responsible for the observed changes in calpain activity. It has previously been demonstrated in an animal model that sepsis stimulates calpain activity in skeletal muscle by decreasing calpastatin activity (174), however, in the study presented in this thesis no significant changes in calpastatin

activity in either exercise group were observed. Such findings may represent a type 2 error as previous work in a porcine model has shown that the use of exogenous calpain inhibitors can reduce infarct size and improve contractility and global haemodynamics following a period of myocardial ischaemia (350). This highlights the need for future studies to further evaluate the role of the calpain system in patients with IC undertaking SET, as the potential exists for the research and development of a pharmacotherapeutic agent with the ability to act as an exogenous calpain inhibitor, which may complement SET through the prevention of potentially detrimental muscle atrophy.

Despite the seemingly obvious link between calpain activity and changes in SMM, results from this study failed to demonstrate a relationship between the two variables. This lack of an observed relationship may be reflective of the fact that change in SMM is multi-factorial. Dietary intake, in particular protein consumption, and REE are both known to impact on SMM (252), however, in both Group A and B, there were no changes in any of these variables over the duration of 12 weeks of SET to explain the observed changes in SMM. The contribution of dietary intake and REE are further discussed below (*See section 6.4.3* Resting Energy Expenditure *and 6.4.4* Dietary Intake*).* Furthermore, numerous hormones including insulin, growth hormone and thyroid hormones are known to regulate body composition (351) and may be impacted on by exercise and IRI.

Whatever the mechanism underlying changes in SMM in response to exercise in these patients with IC, the importance of preserving SMM and the potential detrimental effect of a loss of SMM in the treadmill based training group must not be understated. The central role played by muscle in whole body protein metabolism is particularly important in response to physiological and pathological stress and preservation of

SMM is an important contributor to functional capacity and maintenance of quality of life as well as improving longevity in both healthy individuals and those in a state of chronic disease (252).

A further unique component of this study was the assessment of the calpain system in two separate muscle groups. Current evidence would suggest that calpain activity is intimately related to local conditions (171) and a significant difference was therefore expected between the poorly perfused gastrocnemius muscle and the vastus medialis muscle. There was rationale to believe that the vastus medialis muscle would be relatively well perfused given that the inclusion criteria for the study required the absence of radiologically defined aorto-iliac disease. The lack of an observed significant difference between the two muscle groups was not consistent with this theory and may be explained by the fact that even in the absence of aorto-iliac disease, profunda or small vessel disease may be present in patients with IC, leading to asymptomatic micro- or macro-vascular ischaemic changes in the vastus medialis and other quadriceps muscles with resultant conditions similar to those in the gastrocnemius muscle. In addition, given that different muscle groups have a different structure associated with quite unique functional demands, it is likely that even in the absence of vascular disease, expression and activation of calpain is quite variable (171). Unfortunately, following patient feedback after baseline assessment that two simultaneous biopsies were too much, in order to improve patient compliance, vastus medialis biopsy was only performed at baseline. No data are therefore available to assess the impact of exercise on this muscle group which may be experiencing asymptomatic ischaemic conditions.

The concept of asymptomatic vascular disease in these patients is also relevant when considering the observed differences in SMM between symptomatic and

asymptomatic legs. A large proportion of participants in both groups had radiological evidence of atherosclerotic disease in both legs, even if they were only experiencing unilateral IC. It would therefore seem reasonable to speculate that as a result of these ischaemic conditions, similar changes in body composition, specifically a loss of SMM, may occur in the asymptomatic legs in participants undertaking treadmill based training. Instead, a gain in SMM was observed in these legs. This finding suggests that the presence of clinically significant IRI manifesting as claudication pain is the most important precipitant of a loss in SMM arising from a treadmill based exercise intervention in patients with IC and the presence of occult ischaemia may have minimal impact on body composition in this situation. The reason for the observed gain in SMM in these asymptomatic legs of participants in the treadmill group is unclear. It may however reflect a type 1 statistical error, given that less than half of the total study cohort was experiencing unilateral IC. Further work is therefore required before meaningful conclusions can be made. Given the proposed role of calpain as a regulator of SMM, such further work could involve biopsies of both symptomatic and asymptomatic gastrocnemius muscles with a view to assessing the impact of exercise on calpain activity in each leg.

6.4.2 Fat mass

Also an important determinant of long-term health outcomes is the presence and distribution of FM (252). Abdominal obesity is associated with an increased risk of metabolic syndrome (352), while an insufficient fat mass causes problems such as immunosuppression (353).

It is well established that aerobic exercise can facilitate loss of FM (354), while in studies of resistance training, FM has been observed to either remain stable or decrease (355). It is unclear whether a decrease in FM as a result of resistance training

is a direct effect of such training or related to the gains in SMM with a subsequent increase in REE facilitating loss of FM. Regardless, a significant gain in FM in asymptomatic legs of patients with IC as a result of both exercise regimens was not expected. It is possible that like SMM, such a finding with respect to the asymptomatic legs could be attributed to a type 1 statistical error, although such a finding is not overly concerning given that fat distributed to arms or legs appears to impose little or no risk to future CVD progression or onset (356).

More interesting is the lack of an observed reduction in total FM as a result of the SET in either group. Such a lack of weight loss benefit from exercise interventions may be due to behavioural reasons rather than biological, with evidence suggesting that exercise stimulates dietary intake (357). In conflict to this argument, there was no increase in caloric intake in this study in either group, and in previous work, volunteers in exercise interventions have been found to respond to the increased demands of exercise by reducing body weight and compensating other components of their energy expenditure, rather than increasing dietary caloric intake (358).

Another possible explanation for the lack of reduction in FM is that the fatigue associated with moderate intensity exercise in these patients with IC resulted in them becoming more sedentary when they were not exercising. Although participants were instructed to maintain their usual baseline level of physical activity throughout the SET, physical activity diaries were not part of this study making such a concept difficult to quantify.

In a recent study assessing the impact of low versus moderate to high intensity exercise in overweight men (359), it was concluded that those who exercise at a lower intensity may burn enough calories to precipitate weight loss without feeling the urge

to replace them, either by a more sedentary lifestyle or increased dietary caloric intake. While this may be the case, the priority for SET in patients with IC is improving walking performance and as has been discussed previously (*See section 3.4.2* Volume of exposure to exercise*)* higher intensity exercise is likely to produce superior improvement in walking performance when compared with lower intensity exercise.

6.4.3 Resting Energy Expenditure

The contribution of SMM to REE and vice versa is well recognised and changes in muscle mass and metabolism are therefore reflected in the REE of an individual with potential implications on FM (252). Although there are currently no studies reporting the impact of SET on REE in patients with IC, generally speaking an increase in SMM and associated muscle protein synthesis will lead to an increase in REE due to the energy required to incorporate amino acids into protein (252). Based on the changes in SMM observed in the study presented in this thesis, REE would be expected to decrease in the treadmill training group and increase in the combination group. This was not the case however and REE demonstrated very little change in response to exercise in both groups.

The most plausible explanation for these inconclusive results is the observed variability in REE results which may be masking any potential effect. While undoubtedly part of this variability is attributable to equipment and operator factors, standard calibration techniques were adhered to and it is more likely that our results are reflecting intrinsic variability associated with the assessment of REE.

In addition, consideration must be given to the impact of repeated exposure to IRI on skeletal muscle metabolism. It has been proposed that such exposure can lead to

adaptive changes in skeletal muscle metabolism such as up-regulation of skeletal muscle oxidative enzymes with improvement in oxidative metabolism and more efficient utilisation of energy (164). This is likely a plausible mechanism to explain the improvement in walking performance that has been observed in the literature and may actually serve to lower REE. A similar theory has been used to explain the findings of several studies assessing the impact of high intensity exercise training on REE, in which REE was found to be reduced in spite of increases in SMM.

6.4.4 Dietary Intake

Consistent with findings from Gardner et al(251), the majority of participants with IC in the study presented in this thesis failed to achieve the recommended dietary targets across all key macro and micro nutrients with the exception of protein. This translates to excess total fat, cholesterol and sodium intake and sub-optimal fibre and anti-oxidant intake, the culmination of which is likely to favour the progression of CVD risk factors, ED and subsequently the systemic burden of atherosclerosis. Added to this is inappropriately low consumption of other nutrients such as folate which may precipitate an anaemic state and worsen the symptoms associated with IC.

Importantly, evidence suggests that combined nutrition and exercise interventions in the rehabilitation setting are preferable, with those receiving exercise alone demonstrating greater declines in nutritional status and physical health(259). Furthermore the timing and composition of nutritional support, in particular supplementary protein, is thought to be critical in achieving optimal outcomes for exercise interventions (259).

In the context of muscle wasting and presumed protein turnover induced by increased calpain activity in the treadmill training group in this study, protein supplementation

may be beneficial and lead to further improvement in PFWD while aiding preservation of SMM and subsequently reducing the risk of adverse long-term health outcomes. Although such supplementation seems logical, it must be recognised that a large proportion of patients with IC in the study presented in this thesis actually achieved the recommended daily protein intake. The beneficial effect of additional protein supplementation in these patients therefore remains to be seen and further work is required to assess this.

The findings of the study presented in this thesis would also suggest that the role of nutritional intervention and supplementation may not just be limited to protein. The observed baseline intake of anti-inflammatory and anti-oxidative nutrients such as fibre, folate and certain vitamins, were also below recommended daily levels of intake in many patients with IC. Supplementation with such nutrients may help to protect the endothelium against the harmful pro-inflammatory and pro-oxidative effects of IRI. Whether or not this would serve to directly aid improvements in walking performance remains to be seen, however, the protective effect of such nutritional supplementation on the endothelium may reduce the risk of future CVD events precipitated by repeated exposure to IRI such as the case with treadmill based SET.

Better nutrition education may also facilitate improvement in dietary intake. Enrolment of patients with IC in SET could be considered as an opportunity to seize the teachable moment with the potential to improve both long and short term health outcomes in this cohort of patients who are already at a high risk of future CVD related morbidity and mortality. A targeted intervention in the future to assess such an area may therefore be warranted.

6.4.5 Overview

This work assesses the impact of SET on the nutritional and metabolic status of patients with IC which has not previously been reported in the literature. Although best described as “pilot” data due to the small sample size and potential limitations imposed by study power, the significant changes in SMM and calpain activity would suggest that combination based SET is superior to treadmill training alone with respect to body composition and the potential long-term health benefits associated with preservation and/or gain of SMM. Despite this, such changes were not associated with a significant positive improvement in the primary outcome of walking performance (*See Chapter 3)*, nor were they contributed to by changes in dietary intake or REE. Instead, walking performance was found to improve in association with the presumed detrimental loss of SMM and gain in calpain activity arising from treadmill based SET. Such improvement is likely to be independent of the changes in body composition and again raises the concept that although treadmill training in patients with IC is associated with positive improvements in walking performance, it may have adverse effects on long-term health outcomes. Nutritional supplementation including protein and anti-oxidant vitamins may have a protective effect and should be considered as an adjunctive therapy to SET.

Findings from Chapter 6 have been published in the European Journal of Vascular and Endovascular Surgery and Nutrition Journal:

Delaney CL, Miller MD, Chataway TK, Spark JI. A randomised controlled trial of supervised exercise regimens and their impact on walking performance, skeletal muscle mass and calpain activity in patients with intermittent claudication. Eur J Vasc Endovasc Surg. 2014;47(3):304-310.

Delaney CL, Miller MD, Dickinson KM, Spark JI. Change in dietary intake of adults with intermittent claudication undergoing a supervised exercise program and compared to matched controls. Nutrition Journal 2014, 13:100.

CHAPTER 7: CONCLUSIONS AND IMPLICATIONS FOR FUTURE PRACTICE AND RESEARCH

The topical nature of the study presented in this thesis is highlighted by the following facts: Peripheral arterial disease is a major public health problem with sufferers at a four-fold increased risk of future cardiovascular events and mortality. At initial presentation 20-30% of patients with PAD experience IC and the current gold-standard of treatment to achieve symptomatic improvement in this patient group is a treadmill-based supervised exercise program (7). On this basis, several health services throughout Australia and worldwide are currently in the process of implementing such programs. The resources required for this are substantial and the demand for funding from within state and federal health budgets is increasing. To justify such spending, it is important that maximum benefit is obtained from SET so it is necessary to ensure that participants are demonstrating a clinically meaningful response to the SET without being exposed to an increased risk of future cardiovascular events.

The study presented in this thesis sought to determine whether the additive effect of a supervised exercise regimen combining interval based treadmill training with lower limb resistance exercises would facilitate a greater clinically meaningful response in walking performance, while at the same time limiting exposure to IRI, resulting in a more positive effect on markers of long term cardiovascular health including endothelial function, body composition and systemic inflammatory burden, compared with the recommended treadmill-based training regimen.

Unlike previous work, the study presented in this thesis adds a unique contribution to the literature through the implementation of a combination based exercise group that

has not previously been trialled and an assessment of the systemic and local biological effects of treadmill and combination based exercise regiments in patients with IC.

While neither exercise regimen was superior with respect to the primary outcome of PFWD, treadmill-based SET resulted in a worsening of the biological markers of endothelial function and a relative loss of SMM which may be attributable to the increased level of activity of the proteolytic calpain enzyme. Furthermore, the systemic inflammatory response to exercise in both groups was difficult to interpret, but raised the possibility that SET in patients with IC may induce a pro-inflammatory response, an adaptive anti-inflammatory response or an immunosuppressive response.

These potentially detrimental effects of SET in patients with IC, particularly treadmill-based, have not previously been reported in the literature. Such effects suggest that combination-based SET may be superior to treadmill-based SET with respect to the systemic and local biological effects of such programs.

Throughout this thesis, it has been speculated that increased exposure to IRI in treadmill based training may be responsible for such detrimental effects. Importantly, this raises the question of the safety of exposing patients with IC to exercise interventions that increase the volume of exposure to IRI above the baseline normally experienced by these patients. To put this into perspective, medical practitioners do not encourage cardiac patients experiencing angina pectoris to exercise beyond the threshold of pain due to the risk of myocardial IRI. It has been demonstrated that myocardium reperfused after reversible ischaemia exhibits prolonged depression of contractile function known as myocardial stunning (360). Furthermore, in patients who experience repeated exposure to IRI in the same region of myocardium, irreversible impairment to contractile function can occur, resulting in ischaemic

cardiomyopathy (360). Despite this, patients with IC are encouraged to participate in SET in which treadmill-based exercise to a moderate to extreme level of claudication pain is recommended.

Before it can be concluded that treadmill-based exercise in patients with IC is implicated in adverse outcomes, consideration must be given to whether or not the effects deemed to be potentially detrimental that were observed in the study reported in this thesis, actually translate to a greater incidence of future cardiovascular events, worsening of disease severity and/or an increased risk of premature death. To accurately assess this would require an age and gender matched control group of patients with IC, who are treated with best medical therapy but do not undertake SET. A follow-up period post SET of at least five years would be required to ensure the cardiovascular event rate was such that the study would be adequately powered to observe any change. The lack of a non-exercising control group and the short 12 week duration of the intervention without subsequent follow-up in the study presented in this thesis could therefore be considered a limitation of the study. In the planning stage of this thesis however, there was no evidence in the literature suggesting that SET in patients with IC may be detrimental. On this basis, the assumption was made that exercise in patients with IC is most likely to improve physiological markers of health and promote a healthy lifestyle with global symptomatic improvements. Therefore, for ethical reasons, the study presented in this thesis did not include a non-exercising control group. The duration of the study was designed to be consistent with recommendations provided by international consensus guidelines (7, 26). Also consistent with these recommendations was the choice of a facility-based intervention rather than a home-based intervention. Long term follow-up was deemed outside the scope of this thesis given the short duration of candidature.

If future, large scale studies can confirm the preliminary evidence presented in this thesis that SET, particularly treadmill-based programs, in patients with IC are truly detrimental, then management protocols for the treatment of such patients would need to be reconsidered. As has been discussed previously (*See section 1.1.9* Treatment of Peripheral Arterial Disease*)*, current treatment strategies for patients with IC are twofold: (1) modification of risk profile aimed at preventing disease progression and minimising risk of future CVD events; (2) improving symptomatology and functional status.

Risk factor modification has been achieved through the implementation of "best medical therapy", consisting of pharmacotherapy and lifestyle modification such as smoking cessation and glycaemic control (*See section 1.1.9.1* Strategies for risk factor modification*)*. Supervised exercise training has been prescribed with a view to improving pain free walking performance and functional status. If SET is no longer an option, then the alternative is revascularisation in the form of endovascular therapy or surgery. The risks of such procedures have been discussed *(See section 1.1.9.3* Interventions to provide symptomatic control in PAD and IC*)*, but briefly to reinforce this, blood loss, wound and cardiorespiratory complications are associated with all procedures, while graft or stent thrombosis and thromboembolic events can occur and may compromise limb viability and precipitate progression to CLI and possibly amputation. Furthermore, consideration must be given to the financial implications of such procedures on the health system. In 2009-10, the average cost of an inpatient stay in an Australian public hospital was $4,500 (361). This is likely to be far greater for a surgical patient undergoing an intervention. The TASC II consensus guidelines estimate that the cost associated with percutaneous transluminal angioplasty is $10,000 while for bypass grafting, the cost is approximately $20,000 (7).

Following the acute intervention, the financial and resource burden to the health system is ongoing as patients are condemned to a lifetime of outpatient follow-up and in some cases reintervention to maintain patency of the original intervention. The 12 month primary angiographic patency of a bare metal stent in the superficial femoral artery is 65% (362), while a below knee femoro-popliteal bypass using a saphenous vein graft has a 12 month primary patency of 85% (363). Therefore, to ensure that correctable stenotic lesions are identified and stent/graft patency is preserved, ultrasound and clinical surveillance is routinely undertaken on an ongoing basis.

The risks of undergoing an intervention coupled with the need for ongoing follow-up could be expected to be perceived as quite burdensome by many patients. Interestingly, in the only study to compare QoL in patients who had been randomised to either SET or to receive an endovascular intervention, QoL improved more significantly in those who underwent an intervention (49). Notably, the six month follow-up was relatively short, potentially minimising the burden of long term follow-up on QoL. Also of note, in the same study from Murphy et al (49) treadmill-based SET improved PFWD significantly more than stenting. Other work has demonstrated that QoL was not significantly different between endovascular intervention or SET (135, 364, 365) however, endovascular intervention may be superior to exercise with respect to walking performance in the short-term but this improvement may not be maintained (110). The sustainability of benefit to walking performance following the completion of SET and revascularisation independently requires further investigation.

A formal health economic analysis of SET versus endovascular revascularisation was undertaken by Spronk et al (135) and suggested that with respect to quality adjusted life years (QALY's), there was no significant difference between SET and revascularisation but the cost per QALY was significantly greater with

revascularisation. The limitation of such an analysis was the 12 month follow-up period over which the data was obtained. This is insufficient to enable evaluation of the long-term impact of the potential detrimental effects of SET coupled with the potential beneficial effect of reducing exposure of patients with IC to IRI as a result of revascularisation. It is known that revascularisation can improve endothelial function (366), however whether or not revascularisation has a beneficial effect on systemic inflammatory burden and SMM has not previously been assessed. Future studies to investigate the physiological impact of revascularisation versus SET would therefore be useful, with the view that if revascularisation can improve physiological markers and long-term cardiovascular outcomes compared to SET, then added expenditure per QALY in the first 12 months may be justified.

Consideration should also be given to the concept of revascularisation being used as an adjunctive therapy to SET. Revascularisation prior to SET could be employed to minimise the burden of exposure to IRI during the SET, thus allowing patients with IC to exercise with a reduced risk of detrimental physiological implications. The mild to moderate intermittent claudication trials (MIMIC) has previously investigated the effect of adjuvant endovascular revascularisation (angioplasty) with SET versus SET alone in patients with IC associated with femoro-popliteal disease (367). Although not designed for the purpose of minimising IRI, the MIMIC trial demonstrated that both PFWD and MWD were significantly greater in the group randomised to adjuvant angioplasty immediately following the completion of a six-month SET and after two years of follow-up post SET, compared with those randomised to SET only (367).

While the authors of the MIMIC trial have not proposed any mechanism to explain such observations, they lend significant weight to the potential benefits of revascularisation, either alone or in combination with SET in patients with IC. Once

again, further investigation is required to assess the impact of adjuvant revascularisation on the systemic and local biological responses of patients with IC and additional health economic analysis is also necessary to consider the feasibility of such a combined treatment regimen.

Despite the considerations that exercise in patients with IC, in particular treadmill-based SET, may be detrimental, it is important to remember that the physical and psychological benefits of regular physical activity in adults of all ages is well established (97) and can improve symptomatology and prognosis in those with chronic disease. Therefore, before being conclusive that SET is detrimental to patients with IC, attention must be given to alternative regimens. Many such regimens including progressive whole body resistance training (236), arm-cranking (245) and polestriding (368) have facilitated improvements in walking performance, however, the impact on systemic and local biological responses has not been assessed.

In the study presented in this thesis, an exercise regimen combining treadmill and lower limb resistance training did not impair the health of the endothelium and had a positive impact on SMM. With the exception of its impact on systemic inflammatory burden, which is difficult to interpret, there were no detrimental effects of such SET. Although the combination-based exercise regimen failed to produce any improvement in PFWD in the study presented in this thesis, limitations of this regimen have been discussed *(See Chapter 3)* and include the volume of exposure to each exercise modality and the variability in response to the SET. Importantly, the changes observed in calpain activity, SMM and ADMA levels suggest that there are some beneficial physiological responses occurring as a result of combination based SET.

In conclusion, the study presented in this thesis demonstrates that treadmill-based exercise as a treatment for IC may be detrimental. The prescription of an exercise program to patients with IC is focused on achieving improvement in walking performance, however, this should not come at the expense of physiological changes that are likely to negatively impact on the health of a patient. Findings from this study challenge whether treadmill-based exercise training should remain the recommended treatment for patients with IC. On current evidence, the lack of significant improvement observed in walking performance means that the combination based SET cannot be recommended as a suitable alternative to treadmill-based SET, however, with appropriate modifications to the volume and frequency of training, combination based SET may have a role to play in the future. Further large-scale work is required to address the clinical need to look at better ways to design exercise programs for maximal benefit rather than harm. This should not only focus on the type of exercise, but also the volume and intensity of exposure to exercise. Given the established effect of such exercise on SMM and the poor diet of patients with IC that have been identified in this thesis, consideration could also be given to a multi-disciplinary approach with input from a dietitian. As well, such future work should attempt to confirm or refute the findings of the study presented in this thesis through the assessment of the impact of exercise interventions on the clinical, systemic and local biological responses of patients with IC. In planning such future work, consideration should also be given to long follow-up periods to facilitate the assessment of cardiovascular outcomes and ultimately determine whether or not the changes observed in the study presented in this thesis truly do manifest as detrimental to long-term health outcomes.

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APPENDIX 1 – TIMETABLE OF EXERCISE AND LECTURES

DEPARTMENT OF VASCULAR SURGERY
REPATRIATION GENERAL HOSPITAL
DAWS ROAD, DAW PARK, ADELAIDE SA 5041

Government of South Australia
SA Health

REPATRIATION GENERAL HOSPITAL DAW PARK

FLINDERS MEDICAL CENTRE

CLAUDICANT CLUB PROGRAM
Please report to Reception A in the Rehabilitation Building on arrival.

The Monday education lectures are conducted in either Group Room 3 or 4 in the new Rehabilitation Building at Repatriation General Hospital. They are open to anyone interested in learning more about vascular disease. The 30-minute talks are informal and will provide you with information about "peripheral vascular disease", what causes it and what steps you can take to improve your general health and hopefully slow the progression of the disease. Following the talks you will proceed to the physiotherapy department for the 1-hour gym exercise session. The Thursday physiotherapy sessions are for 1 hour in the gymnasium.

Date	Venue	Speaker	Topic
Monday 10th September (10am)	Group Room 3/4 (then Physio Gym)	Vascular. CNC	Introduction to program + Arterial Disease - risk factors
Thursday 13th September (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 17th September (10.00am)	Group Room 3/4 (then Physio Gym)	Physiotherapist	The benefits of a walking program
Thursday 20th September (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 24th September (10.00am)	Group Room 3/4 (then Physio Gym)	Pharmacist	Medication & arterial disease
Thursday 27th September (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 1st October (10:00am)	**PUBLIC HOLIDAY**	**PUBLIC HOLIDAY**	**PUBLIC HOLIDAY**
Thursday 4th October (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 8th October (10.00am)	Group Room 3/4 (then Physio Gym)	Occupational Therapist	Life style & goal setting
Thursday 11th October (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 15th October (10.00am)	Group Room 3/4 (then Physio Gym)	Podiatrist	The importance of healthy feet
Thursday 18th October (10.30am)	Physio Gym	Physio Gym	Physio Gym

Date	Venue	Speaker	Topic
Monday 22nd October (10.00am)	Group Room 3/4 (then Physio Gym)	Dietician	Diet specific for arterial disease
Thursday 25th October (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 29th October (10.30am)	Physio Gym	Physio Gym	Physio Gym
Thursday 1st November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 5th November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Thursday 8th November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 12th November (10:30am)	Physio Gym	Physio Gym	Physio Gym
Thursday 15th November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 19th November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Thursday 22nd November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Monday 26th November (10.30am)	Physio Gym	Physio Gym	Physio Gym
Thursday 29th November (10.30am)	Physio Gym	Physio Gym	Physio Gym

APPENDIX 2 – AUSTRALASIAN VASCULAR QUALITY OF LIFE QUESTIONNAIRE

Government of South Australia
SA Health

Repatriation General Hospital Daw Park

Flinders Medical Centre

Vascular Surgical Services
Flinders medical Centre
Flinders Drive
Bedford park
SA 5042

www.health.sa.gov.au

Name: *UR Number:*

Date of Birth: *Trial Number:*

Australasian Vascular Quality of Life Questionnaire

Please take some time to answer the following questions. Please select ONE answer that best describes your day to day life.

1. General Health Perceptions

Q1 How has your health been in the last month in comparison with other people you know of the same or similar age?

1. Excellent
2. Very Good
3. Good
4. Fair
5. Poor

Q2 During the last month, how many days have you spent sick in bed?

1. None
2. Between 1 and 5 days
3. Between 6 and 14 days
4. Between 15 and 30 days
5. Everyday

If you have had some vascular surgery, go to Q3a. If not, go to Q3b

Q3a In comparison with your health before vascular surgery, how would you rate your health now?

1. A lot better than before the surgery
2. A little better than before the surgery
3. The same as before the surgery
4. A little worse than before the surgery
5. A lot worse than before the surgery

Q3b In comparison with your health 1 year ago, how would you rate your health now?

1. A lot better than one year ago
2. A little better than one year ago
3. The same as one year ago
4. A little worse than one year ago
5. A lot worse than one year ago

2. Function, Mobility and Pain

Q4 At a steady pace, how far are you able to walk on the flat before becoming short of breath, or experience chest pain, leg pain or another limitation?

1. More than 1km
2. Between 500m and 1km
3. Between 100m and 500m
4. Between 1m and 100m
5. I can't walk/I have pain at rest

Q5 Do you have pain or discomfort in your legs or feet that limits your mobility or disturbs your sleep, ulcers on your feet or have you lost a limb?

1. None of these
2. Leg or foot pain when I walk
3. Leg or foot pain at night
4. I have an ulcer on my foot or leg
5. I've had a limb(s) amputated

Q6 Are you able to do most of the chores around the house and do your grocery shopping?

1. Yes, easily
2. Yes, but I find it difficult
3. Yes, with some assistance
4. Yes, with continuous assistance
5. No, not at al

Q7 Do you suffer from fits, faints, funny turns or memory problems (including epilepsy, transient ischaemic attack, episodes of dizziness, loss of consciousness or stroke)?

1. Never had a non-disabling stroke
2. Occasionally
3. Sometimes
4. Often
5. Continuously/I've had a disabling stroke

Government of South Australia
SA Health

Q8 Are you able to read a magazine or newspaper?
1. Yes, easily
2. Yes, but I find it difficult
3. Yes, with prescription glasses
4. No, but I still have some sight
5. No, I am blind

3. Psycho-Social Aspects

Q9 How often do you see your friends and relatives, or participate in hobbies?
1. Everyday
2. Several times per week
3. Once per week
4. Several times per month
5. Rarely/Never

Q10 Have you felt lonely, unhappy, depressed, or anxious over the past month? In general, have there been any changes in your life that you feel have been detrimental to your quality of life (For example, are there some things you can't do anymore?)

1. No
2. Yes, occasionally
3. Yes, sometimes
4. Yes, often
5. All the time

APPENDIX 3 – PUBLISHED MANUSCRIPTS

A Randomised Controlled Trial of Supervised Exercise Regimens and their Impact on Walking Performance, Skeletal Muscle Mass and Calpain Activity in Patients with Intermittent Claudication

C.L. Delaney [a], M.D. Miller [b], T.K. Chataway [c], J.I. Spark [a,*]

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WHAT THIS PAPER ADDS

Unique to the literature, this study demonstrates that the recommended treatment for intermittent claudication (IC) may be detrimental to long-term health outcomes. Supervised treadmill-based exercise training requires patients to walk beyond the onset of claudication pain with a view that physiological adaptations to the relative ischaemia induced by such training lead to improvements in walking performance. It seems, however, that such repeated ischaemia–reperfusion insults can also lead to a catabolic muscle wasting state, potentially through activation of the calpain system. Findings from this study challenge whether treadmill-based exercise training should remain the recommended treatment for patients with IC.

Objectives: Supervised exercise training (SET) is recommended for patients with intermittent claudication (IC). The optimal exercise programme has not been identified, and the potential adverse effects of exercise on these patients warrant consideration. Calpain proteases have been linked with tissue atrophy following ischaemia–reperfusion injury. High calpain activity may therefore cause muscle wasting in claudicants undergoing SET, and skeletal muscle mass (SMM) is integral to healthy ageing. This study assesses the impact of (1) treadmill-based SET alone; and (2) treadmill-based SET combined with resistance training on pain-free walking distance (PFWD), SMM, and calpain activity.

Methods: Thirty-five patients with IC were randomised to 12 weeks of treadmill only SET (group A), or combined treadmill and lower-limb resistance SET (group B). PFWD via a 6-minute walking test, SMM via dual energy X-ray absorptiometry, and calpain activity via biopsies of gastrocnemius muscles were analysed.

Results: Intention-to-treat analyses revealed PFWD improved within group A (160 m to 204 m, $p = .03$), but not group B (181 m to 188 m, $p = .82$). There was no between group difference ($p = .42$). Calpain activity increased within group A (1.62×10^5 fluorescent units [FU] to 2.21×10^5 FU, $p = .05$), but not group B. There was no between group difference ($p = .09$). SMM decreased within group A (-250 g, $p = .11$) and increased in group B (210 g, $p = .38$) ($p = .10$ between groups). Similar trends were evident for per protocol analyses, but, additionally, change in SMM was significantly different between groups ($p = .04$).

Conclusions: Neither exercise regimen was superior in terms of walking performance. Further work is required to investigate the impact of the calpain system on SMM in claudicants undertaking SET.

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INTRODUCTION

Regular physical activity can improve symptomatology and prognosis in those with chronic disease.[1] In addition to the expected beneficial enhancement of cardiovascular conditioning, consensus guidelines recommend that supervised exercise training (SET) should be made available as a treatment for adults with intermittent claudication (IC) on the basis of high-quality evidence demonstrating improved walking performance following SET of sufficient intensity to induce moderate claudication pain.[2] Despite these recommendations, literature is scant in relation to the long-term health outcomes of patients with IC undertaking SET.

Adverse consequences of SET are possible and warrant investigation. Ischaemia–reperfusion injury (IRI) is

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characterised by an inflammatory cascade and increased microvascular permeability.[3] In IC, low-grade IRI occurs after each bout of exercise-induced pain.[4] This correlates with an increase in pro-inflammatory cytokines and may be responsible for the acute impairment in endothelial function observed after exercise in these patients.[5]

The calpain system is a family of calcium dependent proteases and their endogenous inhibitor calpastatin.[6] Skeletal muscle expresses two ubiquitous calpains (u-calpain and m-calpain) and a muscle-specific calpain (calpain-3).[6] After a stimulus such as exercise, the intracellular calcium concentration increases, leading to calpain activation.[6] Activated, calpains can cause morphological damage to skeletal muscle.[6] Loss of calcium homeostasis during IRI leads to unregulated activation of calcium-dependent enzymes, including the calpain system. This precipitates an extensive proteolytic response leading to cell death.[7]

Evidence from animal models supports a role for calpain-induced muscle atrophy in individuals with peripheral arterial disease (PAD). A rat model of chronic ischaemia demonstrated ischaemia-induced muscle atrophy to be more severe in exercise-trained rats than untrained rats, suggesting that under impaired vascular conditions, exercise training may not be beneficial in maintaining muscle mass.[8] In humans with PAD, lower extremity ischaemia can have a detrimental effect on calf skeletal muscle area when compared to age-matched patients with normal perfusion status.[9] The impact of exercise on muscle mass in these patients is yet to be investigated.

The importance of muscle mass in optimising health outcomes is established. Preservation of muscle mass is an important determinant of ability to perform activities of daily living, quality of life, and survival.[10] Resistance training as an alternative form of SET in patients with IC has previously demonstrated improvements in strength and walking performance comparable to treadmill-based SET.[11] Whether a resistance training SET that preserves skeletal muscle mass (SMM) and limits exposure to IRI translates to an improvement in long-term health outcomes superior to treadmill-based SET is unknown.

The purpose of this study was therefore to investigate the impact of treadmill-based SET alone or in combination with lower limb resistance training on the primary outcome of pain-free walking distance (PFWD) and secondary outcomes, 6-minute walking distance (6MWD), SMM, and calpain activity.

MATERIALS AND METHODS

Sampling

This study was a single-centre randomised controlled trial in which patients presenting to the Southern Adelaide Health Service Vascular Surgery Department between January 2011 and July 2012 were screened for eligibility. The study was approved by the regional ethics committee. All participants provided written informed consent.

Inclusion and exclusion criteria

Patients with a clinical history consistent with IC, ankle brachial pressure index (ABPI) <0.9 and radiographic evidence of infra-inguinal disease in the absence of aorto-iliac disease were eligible for inclusion. Patients were excluded if they (1) had a diagnosis of critical limb ischaemia, (2) had recently (<12 months) undergone peripheral vascular intervention (open surgery or endovascular), or (3) suffered from pre-existing cardio-respiratory morbidities limiting exercise capacity.

Demographics and medical history

Comorbidities, current medications, and smoking status were recorded as part of baseline clinical assessment. Demographic information, including gender and age, was also collected.

Randomisation

Participants were randomised to either group A (12-week treadmill-based SET alone) or group B (12-week treadmill-based SET in combination with lower limb resistance training). Randomisation was performed with a computer-based random number generator using a 1:1 allocation ratio for block sizes which represented the number of participants recruited within each 3-monthly interval.

Sample size

For the primary outcome of PFWD, sample size was estimated based on the baseline PFWD reported by Gardner et al. (172 m)[12] and the mean improvement in PFWD following SET suggested by a recent Cochrane review (82.2 m)[13]—approximately a 30% improvement. Data from McGuigan et al.[14] suggested that improvement in PFWD following a 24-week resistance-based SET was 158%, an additive effect of 128% from resistance-based SET alone. Given that our intervention was 12 weeks long, we halved the expected effect size to 64%, utilised a conservative SD of 75 m ($\alpha = 0.05$, $\beta = 0.8$), and calculated the total sample size required to be $n = 28$ (14 per group).

SET

The SET ran over 12 weeks and consisted of two 60-minute sessions per week supervised by a senior physiotherapist. Prior to the commencement of SET, patients were advised to maintain their baseline level of daily activity for the 12 weeks.

Group A: treadmill-based SET. Participants were advised to walk until claudication pain became unbearable. They then rested until pain resolved and repeated the cycle for the duration of the session. Initial treadmill speed was determined by distance covered in the baseline 6-minute walking test. If the participant did not experience symptoms within 10 minutes of walking, the pace or gradient of walking was increased by 10%.

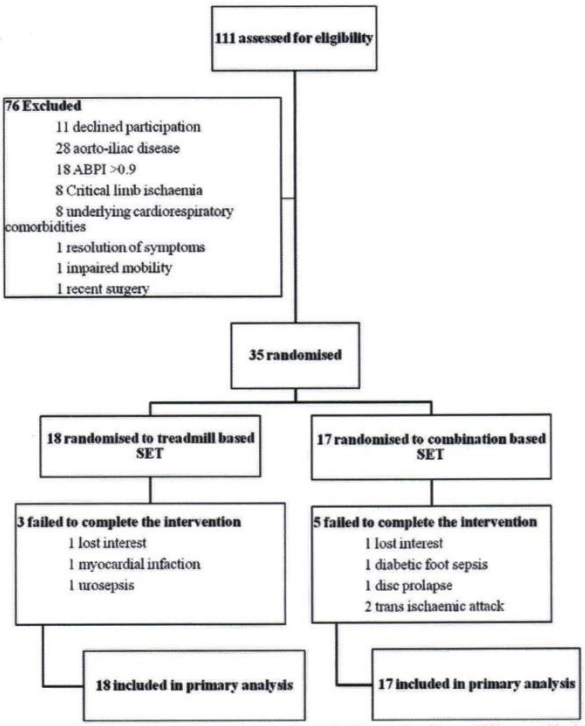

Figure 1. CONSORT diagram outlining the flow of participants through the study. *Note.* ABPI = ankle brachial pressure index; SET = supervised exercise training.

Group B: combination-based SET. Participants were educated to undertake three sets of 8—12 repetitions of hamstring curls, seated calf press, leg press, knee extension, and hip abduction/adduction. For each exercise, participants commenced at the minimum level of resistance. Resistance increased by 5% each time participants achieved 12 repetitions/set Following completion of resistance exercises, participants were asked to walk on the treadmill until the onset of claudication pain only. They then rested until the pain resolved before recommencing resistance exercises. This cycle was repeated for the duration of each session. Initial walking speed and graded increases were determined as described for group A.

Outcome assessments

All measurements were performed within 1 week prior to the commencement of SET and 1 week after completion of SET. Assessors were blinded to allocation.

Six-minute walk test. A standardised protocol was followed. Participants rested for 30 minutes before walking, self-paced along an indoor, flat, straight walkway marked at regular intervals. PFWD was recorded as the distance at which participants first reported claudication pain. The distance covered over the course of the 6 minutes was recorded as the 6MWD.

Muscle biopsy technique. Ultrasound-guided needle biopsy of symptomatic medial gastrocnemius muscle was performed using a disposable core biopsy instrument. In cases where both legs were symptomatic, the leg with the lowest resting ABPI was chosen. Muscle samples were immediately snap frozen in liquid nitrogen and stored at −80°.

Calpain and calpastatin activity. For each sample, skeletal muscle extract (containing 50 µg protein) was added to a microtitre plate well and analysis of m-calpain and calpastatin activity was undertaken according to a previously validated technique.[15] All samples were assayed in duplicate. Calpain activity was expressed as fluorescent units (FU) and calpastatin activity as % inhibition of calpain activity.

SMM. Whole body and regional body composition were estimated using dual energy X-ray absorptiometry (DEXA) with the automated reporting bone densitometry software. Appendicular lean soft tissue mass from DEXA was used to determine SMM according to an established equation.[16] For the calculation and analysis of SMM in symptomatic and asymptomatic legs, given that some participants suffered unilateral claudication and others bilateral claudication, data from each leg were considered as separate entities.

Adherence to SET. Attendance at each exercise session was recorded and satisfactory completion of the SET was determined to be attendance at >80% of sessions.

Data analysis

The primary analysis was undertaken on an intention-to-treat (ITT) basis with those lost to follow-up at 12 weeks having their baseline measure carried forward. For per-protocol analysis, all participants achieving <80% adherence to the allocated SET and those that were lost to follow-up were excluded from the analysis. SPSS v. 19 was used to perform statistical analyses. Continuous data are reported as mean (SD) and categorical data as *n* (%). Differences within groups for continuous data were tested using paired samples *t* tests and change from baseline to 12 weeks between groups using independent samples *t* tests. Differences between groups for categorical data were tested using chi-square test. Two-way analysis of covariance was applied to the primary outcome (PFWD) with adjustment for age and gender, and testing for an interaction effect between group and gender. Statistical significance of $p < .05$ was assumed.

RESULTS

Thirty-five of 46 patients with IC meeting the inclusion criteria agreed to participate in the study, 76% consent rate. Fig. 1 illustrates the flow of participants from enrolment into the study to follow-up at 12 weeks on completion of the allocated SET.

At baseline there was no significant difference between groups in lowest resting ABPI; mean 0.71 (SD 0.23) and mean 0.72 (SD 0.15) for group A and group B respectively ($p = .97$). On commencement of the SET, all participants in each group were on antiplatelet and lipid-lowering agents, while 15 (83%) and 15 (88%) were on anti-hypertensive agents in group A and group B respectively ($p = .68$). Table 1 highlights baseline demographic characteristics and medical history of participants according to group allocation. There were no statistically significant differences between groups identified according to allocation for age, gender, body mass index, smoking status, or medical history.

Table 2 highlights the change in the outcome measures from baseline to 12 weeks according to allocation, for both ITT and per-protocol analyses. There were no statistically significant differences observed between groups at baseline.

PFWD increased significantly between baseline and 12 weeks for group A. A small increase was achieved between baseline and 12 weeks for group B; however, this did not achieve statistical significance and there was no between group difference for the increase observed in PFWD. After adjustment for age and gender there remained no significant difference between the two groups (group: $F[1,27] = 1.832$, $p = .187$; gender: $F[1,27] = .144$, $p = .707$; age: $F[1,27] = .671$, $p = .420$) nor was there any interaction between group and gender ($F[1,27] = 1.619$, $p = .214$).

Table 1. Baseline characteristics between participants in the treadmill-only supervised exercise training (SET) and the treadmill and resistance exercise SET. Data presented as *n* (%) unless otherwise stated.

Demographics and medical history	Treadmill only ($n = 18$)	Treadmill and resistance training ($n = 17$)	*p-value*
Mean (SD) age, y	73.4 (9.1)	69.4 (9.6)	0.22
Male:female ratio	12:5	13:4	0.52
Current/ex-smoker	13 (72)	13 (76)	0.77
Mean (SD) body mass index, kg/m^2	27.0 (4.3)	29.0 (5.6)	0.39
Ischaemic heart disease	6 (33)	8 (47)	0.41
Dyslipidaemia	16 (89)	16 (94)	0.58
Hypertension	14 (78)	15 (88)	0.41
Diabetes mellitus	6 (33)	10 (59)	0.13

6MWD improved in both groups between baseline and 12 weeks; however, this only achieved statistical significance for those participants allocated to group A. There were no between group differences identified.

Calpain activity increased in group A and decreased in group B between baseline and 12 weeks for ITT analyses, reaching statistical significance within group A only. The between group comparison did not achieve statistical significance and when per-protocol analyses were performed, the within and between group differences were similar in magnitude and statistical significance. Calpastatin activity did not achieve statistical significance within or between groups. Coefficient of variance was 5.8% and 39.8% for calpain and calpastatin activity respectively.

SMM demonstrated a non-significant decrease between baseline and 12 weeks for those allocated to group A. A non-significant increase in SMM was observed between baseline and 12 weeks for those allocated to group B. On per-protocol analysis statistical significance was achieved for between group differences.

For the symptomatic leg, SMM decreased non-significantly for those allocated to group A. In contrast, SMM of the symptomatic leg for those allocated to group B increased in both ITT and per-protocol analyses, and while this within group change from baseline to 12 weeks did not reach statistical significance on ITT analysis, it was statistically significant on per-protocol analysis ($p = .04$). These results translated into a borderline statistically significant difference between groups on ITT analysis ($p = .05$) and a clear significance on per-protocol analysis ($p = .02$).

Table 2. Comparison of baseline, 12-week, and absolute change for outcomes between participants in the treadmill-only supervised exercise training and the treadmill and resistance exercise training. Data presented as mean (SD) unless otherwise stated. For intention-to-treat (ITT) purposes *n*-values vary according to whether last outcome carried forward was used or where no baseline measure was available the follow-up value remained missing.

Outcome	Treadmill only				Treadmill and resistance training				Between
	Baseline	12 weeks	Change	*p*	Baseline	12-weeks	Change	*p*	group *p*
ITT[a]									
Pain-free walking distance, m	160 (84)	204 (97)	44 (80)	.03	181 (90)	188 (109)	7 (135)	.82	.42
6-minute walking distance, m	354 (99)	386 (85)	32 (47)	.01	368 (69)	419 (168)	51 (165)	.21	.67
Calpain activity, FU ($\times 10^5$)	1.62 (0.95)	2.21 (1.26)	0.59 (1.24)	.05	1.67 (0.90)	1.45 (1.39)	−0.22 (1.43)	.55	.09
Calpastatin activity, FU ($\times 10^6$)	2.74 (1.05)	3.13 (1.11)	0.39 (1.45)	.27	2.93 (1.00)	2.68 (0.92)	−0.25 (1.39)	.50	.21
SMM,[b] kg	23.72 (6.77)	23.47 (6.62)	−0.25 (0.55)	.11	25.43 (5.42)	25.64 (5.52)	0.21 (0.81)	.38	.10
Symptomatic leg SMM,[c] kg	7.90 (2.16)	7.80 (2.10)	−0.10 (0.28)	.10	8.38 (1.65)	8.52 (1.80)	0.14 (0.47)	.19	.05
Asymptomatic leg SMM,[d] kg	7.62 (1.65)	7.81 (1.92)	0.19 (0.28)	.21	7.86 (1.40)	7.96 (1.31)	0.10 (0.20)	.19	.58
Per protocol									
Pain-free walking distance,[e] m	170 (82)	221 (97)	50 (78)	.03	170 (90)	188 (127)	18 (147)	.67	.59
6-minute walking distance,[e] m	371 (94)	409 (70)	38 (49)	.01	358 (68)	439 (188)	81 (189)	.16	.47
Calpain activity,[f] FU ($\times 10^5$)	1.35 (0.59)	2.11 (1.25)	0.76 (1.37)	.05	1.60 (0.99)	1.45 (1.50)	−0.15 (1.55)	.75	.13
Calpastatin activity,[f] FU ($\times 10^6$)	2.64 (1.10)	3.18 (1.19)	0.54 (1.69)	.28	2.83 (1.02)	2.62 (0.95)	−0.21 (1.53)	.65	.27
SMM,[g] kg	22.84 (6.93)	22.64 (6.74)	−0.20 (0.59)	.28	25.24 (5.91)	25.64 (6.05)	0.40 (0.70)	.09	.04
Symptomatic leg SMM,[h] kg	7.96 (2.29)	7.88 (2.21)	−0.08 (0.28)	.271	8.35 (1.81)	8.59 (1.97)	0.24 (0.40)	.04	.02
Asymptomatic leg SMM,[i] kg	8.10 (1.48)	8.40 (1.64)	0.30 (0.16)	.03	7.70 (1.44)	7.85 (1.38)	0.15 (0.16)	.05	.19

Note. FU = fluorescent unit; SMM = skeletal muscle mass.

[a] Five and three participants in groups A and B, respectively, did not complete the study. Of these, three and two, respectively, returned for follow-up assessment and hence contributed data for ITT purposes. Two and one, respectively, did not return for a follow-up and hence had their baseline data carried forward to enable ITT analysis.

[b] $n = 14$ in group A and $n = 13$ in group B.

[c] $n = 23$ in group A and $n = 18$ in group B.

[d] $n = 5$ in group A and $n = 8$ in group B.

[e] $n = 15$ in group A and $n = 12$ in group B.

[f] $n = 14$ in group A and $n = 12$ in group B.

[g] $n = 11$ in group A and $n = 11$ in group B.

[h] $n = 19$ in group A and $n = 16$ in group B.

[i] $n = 4$ in group A and $n = 7$ in group B.

For the asymptomatic leg, there was an increase in SMM from baseline to 12 weeks within groups, which achieved statistical significance on per-protocol analysis ($p = .03$ and $p = .05$ for group A and group B respectively). There was no between group difference detected in either ITT or per-protocol analyses.

DISCUSSION

The impact of different modalities of SET on body composition and the role of calpain proteases in augmenting such an impact has not previously been investigated in patients with IC. Furthermore, the quest for the most effective exercise regimen to maximise improvements in walking performance in these patients is ongoing.

This study has identified that conventional SET recommended to those with IC can increase calpain proteolytic activity with a relative reduction of SMM, which may be detrimental to long-term health outcomes. While this study was unable to confirm that a combined treadmill and resistance training programme can achieve the equivalent gains in walking performance as a programme of treadmill training alone, it does highlight the need for greater scrutiny of exercise programmes in these patients where delay in disease progression is critical.

Treadmill-based SET until moderate claudication pain is recommended for IC;[2] however, the most effective exercise regimen remains unclear. In our study, exercise to the extreme of claudication pain was prescribed in an attempt to maximise the benefits derived from the mechanisms proposed to explain the observed improvements in walking performance. These include improved nitric oxide bioavailability and endothelial function,[17] and skeletal muscle metabolic adaptations to ischaemic conditions.[17] Unfortunately, such mechanisms are generally activated by the IRI accompanying the claudication pain induced by treadmill-based exercise in these patients. This may stimulate unwanted mechanisms such as a pro-inflammatory response and catabolic state.

Similar to treadmill-based training, resistance training may improve walking performance in patients with IC.[11] Overall, improvements in cardiovascular conditioning and a gain in SMM leading to greater functional capacity are likely responsible for this. A comparative group consisting of lower-limb resistance training combined with treadmill-based training was therefore chosen for this study as it was anticipated that the additive effect of mechanisms responsible for improvement in both treadmill and resistance training may lead to greater improvements in walking performance.

Reducing the exposure to IRI was also anticipated to have a beneficial effect on SMM and the calpain system.

Despite this, although a significant improvement in walking performance was observed within the treadmill-only exercise group, this was not superior to the non-significant change observed in participants undertaking a combination of treadmill and lower-limb resistance training. While we were unable to demonstrate a superior effect of the combined resistance training and treadmill training on PFWD, it is expected that this is directly related to the greater-than-expected variability in this outcome and associated type 2 error rather than an ineffectual exercise regimen. Variability in response to SET has been previously demonstrated.[18] Reasons for such variability are poorly understood, but is likely multifactorial and determined by the mechanism(s) of action underlying the potential beneficial effects of exercise in IC. Genetic factors are likely to be involved in angiogenic or metabolic adaptations, while psychological factors may contribute. Another factor that may have contributed to the lack of observed improvement in the combination-based exercise group is that in an attempt to successfully combine two exercise modalities the participants in the combination group were not exposed to a sufficient volume of either treadmill or resistance training to enable an adaptive response to either modality to occur. A similar conclusion was reached by Treat-Jacobsen et al.,[18] who failed to demonstrate an additive effect of combining arm-cranking and treadmill training. Consideration should also be given to the fact that although participants were asked to maintain their baseline level of physical activity, it was not monitored and may be a confounding factor.

In contrast to walking performance, and suggesting a beneficial effect of the combination-based SET, are the results pertaining to SMM, which demonstrate a superiority of a combination-based SET over a treadmill-based programme. It seems that the impact of a SET on SMM in IC is dependent on modality of exercise and may have a different effect on symptomatic compared with asymptomatic legs. Treadmill-only training resulted in a loss of SMM in symptomatic legs that was significantly different to the gain in SMM observed in symptomatic legs after combination training. These findings were also reflected in total body SMM. In contrast, gains in SMM were observed in asymptomatic legs, irrespective of exercise modality.

A mechanistic explanation for these changes in SMM may lie with the calpain system. The proteolytic activity of the calpain system is implicated in muscle apoptosis and changes in muscle fibre morphology.[6] We know that SET in claudicants results in changes in muscle fibre types,[17] and an increase in calpain activity, as was observed with treadmill-based SET, may be the intracellular mechanism responsible for such change and, subsequently, the relative loss of SMM observed in this group. The calcium-dependent calpain system becomes activated when calcium levels are physiologically increased by stimuli, such as IRI,[6] and the observed increase in calpain activity may be precipitated by a greater exposure to IRI induced by repetitive bouts of treadmill exercise.

In the combination training group, lack of a significant effect on calpain activity is likely due to an interplay of factors counteracting each other. Resistance training has been shown to up-regulate expression of calpain and, to a greater extent, its endogenous inhibitor calpastatin.[19] While not previously demonstrated, a potential decrease in calpain proteolytic activity may ensue. Furthermore, the discussion above would suggest that exposure to a degree of IRI induced by the treadmill-based component of the combination-based SET is likely to increase calpain activity. The net effect of such a programme is a non-significant decrease in calpain activity. In this way, other mechanisms responsible for gains in SMM associated with resistance training and the protective effect of aerobic training against age-related reductions in SMM were allowed to proceed without conflict from the calpain system and may explain the beneficial gain in SMM observed in this group of participants. Support for this concept is also provided by the significant gain in SMM that was observed in the asymptomatic legs of participants within both groups. Although many of these limbs demonstrated radiological evidence of atherosclerotic disease, it seems that the presence of clinically significant IRI manifesting as claudication pain and potentially driving activation of the calpain system is the most important precipitant of a loss of SMM arising from a treadmill-based SET.

It has been demonstrated in an animal model that sepsis stimulates calpain activity in skeletal muscle by decreasing calpastatin activity.[20] Our study did not observe any significant changes in calpastatin activity in either exercise group; however, this may be a reflection of the high coefficient of variance that was recorded. Future work is required to further evaluate the role of calpastatin in exercising claudicants.

Whatever the mechanism underlying changes in SMM in response to exercise in claudicants, the importance of preserving SMM and the potential detrimental effect of a relative loss of SMM in the treadmill-based training group must not be understated. Whether this overrides the expected cardiovascular benefits of exercise remains to be seen and should be the subject of large-scale trials with long-term follow-up; however, the central role played by muscle in whole body protein metabolism is important in response to physiological and pathological stress. Preservation of SMM is an important contributor to functional capacity and maintenance of quality of life, as well as improving longevity in healthy individuals and those with chronic disease.[10]

Although small and best described as a pilot study, this work demonstrates that treadmill-based exercise as a treatment for IC may be detrimental owing to the induction of a catabolic muscle wasting state. The prescription of an exercise programme to claudicants is focused on achieving improvement in walking performance; however, this should not come at the expense of physiological changes that are likely to negatively impact on the health of a patient. Findings from this study challenge whether treadmill-based exercise training should remain the recommended

treatment for patients with IC. On current evidence, the lack of significant improvement observed in walking performance means that we are unable to recommend the combination-based exercise regimen as a suitable alternative to treadmill training alone. Despite this, the superiority of this regimen compared with the treadmill-based regimen with respect to calpain activity and SMM would suggest that, with appropriate modifications, it may have a role to play in the future. Further large-scale work is required to address the clinical need to look at better ways to design exercise programmes for maximal benefit rather than harm. In planning such future work, consideration should also be given to long follow-up periods to facilitate the assessment of cardiovascular outcomes and ultimately determine whether or not the changes observed in this current study truly do manifest as detrimental to long-term health outcomes.

CONFLICT OF INTEREST

None.

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The impact of different supervised exercise regimens on endothelial function in patients with intermittent claudication

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Abstract

Background and objectives: The impact of supervised exercise training on endothelial function in patients with intermittent claudication is unclear. This study assesses the impact of treadmill-based supervised exercise training alone or in combination with resistance training on pain free walking distance, flow-mediated dilatation, reactive hyperaemia index, nitric oxide and asymmetric dimethylarginine.

Methods: Thirty-five patients with intermittent claudication were randomised to 12 weeks of treadmill-only supervised exercise training (Group 1) or a combination of treadmill and lower-limb resistance supervised exercise training (Group 2). Pain free walking distance was assessed by six-minute walk test. Endothelial function was assessed by brachial artery flow-mediated dilatation, reactive hyperaemia index and serum analysis of asymmetric dimethylarginine and nitric oxide.

Results: Pain free walking distance improved within Group 1 (160 m to 204 m, $p = 0.03$) but not Group 2 (181 m to 188 m, $p = 0.82$), no between group difference. No significant change in flow-mediated dilatation or reactive hyperaemia index in either group. Nitric oxide decreased in Group 1 (15.0 μmol/L to 8.3 μmol/L, $p = 0.003$) but not Group 2 (11.2 μmol/L to 9.1 μmol/L, $p = 0.14$), $p = 0.07$ between groups. Asymmetric dimethylarginine decreased in Group 2 (0.61 μmol/L to 0.56 μmol/L, $p = 0.03$) but not Group 1 (0.58 μmol/l to 0.58 μmol/L, $p = 0.776$), no between group difference.

Conclusion: Supervised exercise training does not improve endothelial function as measured by flow-mediated dilatation, reactive hyperaemia index and nitric oxide bioavailability.

Keywords

Peripheral arterial disease, intermittent claudication, endothelial function, supervised exercise training

Introduction

Supervised exercise training (SET) is recommended for patients with Intermittent Claudication (IC) with meta-analyses demonstrating significant improvement in walking performance following SET.[1] Such a clinical response to exercise in claudicants is likely due to an array of systemic and local biological adaptations that occur during the period of exercise. Several mechanisms have been proposed and include stimulation of a pro-angiogenic response[2] and skeletal muscle metabolic adaptations to ischaemic conditions[3] allowing improved tolerance of exercise and better functional results. Evidence also suggests that exercise programs spanning a range of demographics result in improvement of vascular endothelial function and may be responsible for the cardiovascular health benefits associated with exercise training.[4] Given that a dysfunctional endothelium has been proposed as the mechanism linking cardiovascular risk factors to established disease[4] and its onset has been deemed a sentinel event in the progression to atherosclerosis[5] it is not surprising that improvements in nitric oxide bioavailability and endothelial function have also been proposed as a mechanism by which SET improves walking performance in claudicants.[6]

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The vascular endothelium plays a key role in regulating inflammation, coagulation, vasomotor tone and smooth muscle cell proliferation.[5,6] Endothelial dysfunction (ED) is a state of impairment of these regulatory functions, characterised by an impaired endothelium dependent vasodilatory response induced by changes in flow or stimuli.[5,6] It can be assessed by various techniques including flow-mediated dilatation (FMD) and reactive hyperaemia peripheral arterial tonometry (RH-PAT).[7] Nitric oxide (NO) is a potent vasodilatory agent secreted by the endothelium whose pleiotropic effects include the inhibition of both platelet aggregation and inflammation.[8] Reduced levels of NO play a key role in the pathogenesis of ED and help to explain the association of ED with a pro-thrombotic and systemic pro-inflammatory state.[8] Asymmetric dimethylarginine (ADMA) is released by the methylation of arginine residues in proteins by protein arginine methyltransferases. It can act as an endogenous, competitive inhibitor of the L-arginine required for NO production.[8] Raised levels of ADMA are therefore likely to inhibit NO synthesis and impair endothelial function and thus promote atherosclerosis. Elevated plasma levels of ADMA have been reported in patients with atherosclerosis.[9]

Exercise in healthy individuals has been shown to augment blood flow and intravascular shear stress, resulting in increased NO production and improved endothelial function.[6] There is evidence demonstrating improvements in endothelial function following SET in claudicants, manifesting as improvement in FMD and NO bioavailability.[10,11] Despite this, the role of ADMA in regulating such a response is rarely reported, while the impact of SET in claudicants on the reactive hyperaemic index (RHI) has not previously been assessed. Furthermore, only once in the literature has endothelial function been considered following an exercise modality other than treadmill training.[10] McDermott et al.[10] demonstrated improvement in FMD following 24 weeks of supervised lower limb resistance training that was equivalent to the improvement associated with supervised treadmill training. Whether or not there exists an additive benefit to endothelial function by combining both regimens warrants exploration. Any such benefit would be significant considering the favourable effect of endothelial function on long-term cardiovascular outcomes.[12]

In adults with IC, the aim of this study was to determine whether a 12-week SET with interval treadmill and lower-limb resistance training produces more clinically and statistically significant improvements than a supervised treadmill exercise program alone and is, therefore, superior with respect to our primary outcome of pain free walking distance (PFWD) and secondary outcomes relating to endothelial function, including FMD, RHI, NO and ADMA.

Subjects and methods

Subjects

Sampling. This is a single centre randomised controlled trial in which consecutive patients presenting to the Southern Adelaide Health Service Vascular Surgery Department between January 2011 and July 2012 were screened for eligibility. The study was approved by the Southern Adelaide Human Research Ethics Committee. All participants provided written informed consent.

Inclusion and exclusion criteria. Patients with a clinical history consistent with IC, ankle brachial pressure index (ABPI) <0.9 and radiographic evidence of infra-inguinal disease in the absence of aorto-iliac disease were eligible for inclusion. Patients were excluded if they (1) had a diagnosis of critical limb ischaemia, (2) had recently (<12 months) undergone peripheral vascular intervention (open surgery or endovascular), or (3) suffered from pre-existing cardio-respiratory morbidities limiting exercise capacity.

Demographics and medical history. Comorbidities and smoking status were recorded as part of baseline clinical assessment. Demographic information including gender and age was also collected.

Randomisation. Participants were randomised to either Group 1: 12-week treadmill-based SET alone or Group 2: in combination with lower limb resistance training. Randomisation was performed with a computer-based random number generator (Excel 2010; Microsoft, Seattle, Washington, USA) using a 1 : 1 allocation ratio for block sizes which represented the number of participants recruited within each 3-month interval.

Sample size. For the primary outcome of PFWD, sample size was estimated based on baseline PFWD reported by Gardner et al.[13] (172 m) and the mean improvement in PFWD following SET suggested by the recent Cochrane review (82.2 m),[14] approximately a 30% improvement. Data from McGuigan et al.[15] suggested that improvement in PFWD following a 24-week resistance-based SET was 158%, an additive effect of 128% from the resistance-based SET alone. Given our intervention was 12 weeks, we halved the expected effect size to 64%, utilised a conservative SD of 75 m, a Type 1 error of alpha = 0.05 and Type 2 error of beta = 0.8 and calculated the total sample size required to be n = 28 (14 per group).

Interventions

Supervised exercise training (SET). International consensus guidelines recommend that exercise sessions are conducted over as 12-week period.[1] The SET, therefore, ran over 12 weeks and consisted of two 60-minute sessions per week supervised by a senior physiotherapist. Prior to the commencement of SET, patients were advised to maintain their baseline level of daily activity for the 12-week duration.

Treadmill-based SET (Group 1). Participants were advised to walk until claudication pain became unbearable. They then rested until pain resolved and repeated the cycle for the duration of the session. Initial treadmill speed was determined by distance covered in the baseline six-minute walk test. If the participant did not experience symptoms within 10 min of walking, the pace or gradient of walking was increased by 10%.

Combination based SET (Group 2). Participants were educated to undertake three sets of 8–12 repetitions of hamstring curls, seated calf press, leg press, knee extension and hip abduction/adduction. For each exercise, participants commenced at the minimum level of resistance. Resistance increased by 5% each time participants achieved 12 repetitions/set. Following completion of resistance exercises, participants were asked to walk on the treadmill until the onset of claudication pain only. They then rested until the pain resolved before recommencing resistance exercises. This cycle was repeated for the duration of each session. Initial walking speed and graded increases were determined as described above for Group 1.

Outcome assessments

All assessments were undertaken in the afternoon (between 1300 h and 1700 h) before and after completion of the 12-week SET. Assessors were blinded to the allocated intervention. Walking performance was assessed at a separate time to measures of endothelial function to avoid the confounding effect of exercise on endothelial function.[16] Prior to assessment of endothelial function, subjects were requested to refrain from high fat foods, caffeine, tobacco and alcohol for at least eight hours prior and to avoid strenuous exercise on the morning of the test.

Six-minute walk test. A standardised protocol was followed in which participants rested for 30 min before walking, self-paced along an indoor, flat, straight walkway marked at regular intervals. PFWD was recorded as the distance at which participants first reported claudication pain. Participants whose PFWD improved were considered to be responders to SET. Non-responders to SET were those who demonstrated no improvement or worsening in PFWD.

Quality of life (QoL). The Australasian Vascular Quality of Life Index (AUSVIQUOL) is a validated tool for the assessment of QoL of patients with PAD in the clinical setting. It consists of 10 questions addressing general health perceptions, function mobility and pain and psychosocial aspects of health. Participants were asked to complete this questionnaire independently and a score out of 50 was recorded.

Brachial artery ultrasound flow-mediated dilatation. Flow-mediated dilatation (FMD) was assessed using techniques consistent with published guidelines.[7,17] With the participant supine, a blood-pressure cuff was placed around the forearm and the ultrasound transducer was placed on the arm proximal to the elbow. A 3 lead ECG was connected to the ultrasound machine to enable display of the cardiac cycle.

A 30 sec baseline period of scanning of the brachial artery was recorded prior to cuff inflation. The cuff was then inflated to 250 mmHg for 5 min to achieve total brachial artery occlusion. Recording recommenced at 15 sec post occlusion and continued for 3 min.

Measurement was undertaken by automated edge detection software (Brachial Artery Analyser, MIA-LLC, Coralville, USA). All measurements were obtained during diastole. The FMD was calculated using the equation

$$\text{FMD}\,(\%) = \left[\frac{(\text{peak diameter} - \text{baseline diameter})}{\text{baseline diameter}}\right] \times 100$$

Reactive hyperaemic peripheral arterial tonometry (RH-PAT). RH-PAT was performed using an EndoPAT device (Itamar Medical Ltd, Caesarea, Israel), following the manufacturer's guidelines. The occlusion cuff was placed above the elbow and fingertip plethysmography probes were placed on the index fingers of each hand. The pulse wave amplitude was continuously recorded by the device. The test commenced with a 5 min baseline period, followed by 5 min with the cuff inflated to 250 mmHg to achieve total brachial artery occlusion and then a final 5 min period of reactive hyperaemia following the cuff release. The recordings were analysed using the device's proprietary software and the reactive hyperaemia index (RHI) was calculated using the method previously described by McCrea et al.[18]

Serum biomarkers (nitric oxide and asymmetric dimethylarginine). Venepuncture was performed and samples were immediately placed on ice before centrifugation of

samples took place within 1 h of collection. Serum samples were stored at −80°C before analysis of serum was undertaken using commercially available and previously validated ELISA kits (Total Nitric Oxide Assay Kit, Thermo Fisher Scientific, Illinois, USA and ADMA Human ELISA Kit, Enzo Life Sciences, New York, USA). The NO assay measured levels of nitrite and nitrates (stable metabolites of NO) and converted these to a total NO concentration.

Statistical analyses

The primary analysis was undertaken on an intention to treat (ITT) basis with those lost to follow up at 12 weeks having their baseline measure carried forward. For per protocol analysis, all participants achieving less than 80% adherence to the allocated supervised exercise program and those that were lost to follow up were excluded from the analysis. Statistical Package for Social Sciences version 19 (SPSS Inc, Chicago IL, USA) was used to perform statistical analyses. Continuous data are reported as mean (SD) or median (IQR) according to normality and categorical data as n (%). Differences within groups for continuous data were tested using paired samples t-test or Wilcoxon Signed Rank test and change from baseline to 12 weeks between groups using independent samples t-test or Mann-Whitney U test. Differences between groups for categorical data were tested using X^2. Relationships between NO and ADMA were assessed using Spearman correlation coefficient. Statistical significance of $P < 0.05$ was assumed.

Results

Participants

One hundred and eleven participants were assessed for eligibility. The flow of participants through the study is highlighted in Figure 1. Participants' clinical characteristics at baseline are summarised in Table 1.

Pain free walking distance (PFWD) (Table 2)

There was no significant difference in baseline PFWD between groups ($P=0.593$). PFWD increased significantly between baseline and 12 weeks for Group 1, both on ITT and per-protocol analysis, however, there was no between group difference observed.

Quality of Life (QoL) (Table 2)

A lower value represents improvement in QoL. Significant improvements in QoL score were reported in both Group 1 ($P=0.01$) and Group 2 ($P=0.01$). The improvement noted between groups was comparable with no statistically significant difference ($P=0.18$).

Flow-mediated dilatation (FMD) (Table 2)

There was no significant difference in baseline FMD between groups ($P=0.398$). Change in FMD from baseline to 12 weeks was not statistically significant between groups.

Reactive-hyperaemia peripheral arterial tonometry (RH-PAT) (Table 2)

There was no significant difference in baseline RHI between groups ($P=0.193$). There was no change in RHI from baseline to 12 weeks observed in either group.

Nitric oxide (NO) (Table 2)

There was no significant difference in baseline NO between groups ($P=0.205$). With per protocol analysis, NO activity was significantly reduced as a result of the SET in Group 1 ($P=0.002$) but not Group 2. This also produced a statistically significant between group difference ($P=0.040$). With ITT analysis, NO remains lower after intervention in Group 1 ($P=0.003$) and not Group 2, however, while there is a trend towards a significant difference between groups, this did not reach statistical significance ($P=0.066$).

Asymmetric Dimethylarginine (ADMA) (Table 2)

There was no significant difference in baseline ADMA between groups ($P=0.709$). After the 12-week SET, ADMA remained virtually at baseline in Group 1. In contrast, the level of ADMA was significantly reduced in Group 2 participants with ITT analysis ($P=0.028$), while the reduction observed from per-protocol analysis is also significant ($P=0.05$). These differences were not statistically significant between groups, $P=0.193$ and $P=0.236$ for ITT and per-protocol analysis respectively. No relationship was observed between the change in ADMA and NO activity in Group 1 ($r=0.071$, $P=0.781$) or Group 2 ($r=0.087$, $P=0.740$).

Responders vs non-responders

There were 10 (67%) participants in Group 1 who demonstrated improvement in PFWD and five (42%) in Group 2. Within each group, there was no significant change observed in FMD, RHI, NO or ADMA between responders and non-responders. Similarly, no change was observed when responders from both

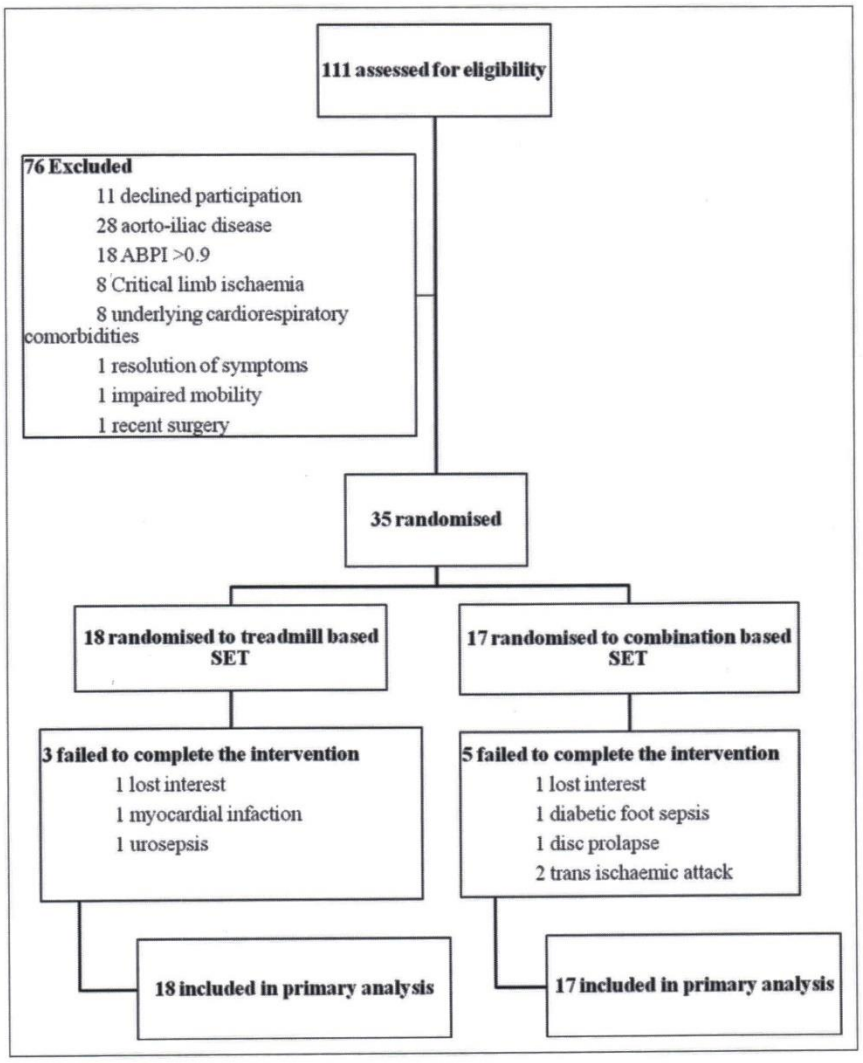

Figure 1. CONSORT diagram outlining the flow of participants through the study.
ABPI: ankle brachial pressure index; SET: supervised exercise training.

Table 1. Baseline characteristics between participants in the treadmill only supervised exercise training (Group 1) and the treadmill and resistance supervised exercise training (Group 2). Data presented as n (%) unless otherwise stated.

Demographics and medical history	Group 1: treadmill only (n = 18)	Group 2: treadmill and resistance training (n = 17)	*P*-value
Mean (SD) age (years)	73.4 (9.1)	69.4 (9.6)	0.22
Male:female ratio	12:5	13:4	0.52
Caucasian	18 (100)	17 (100)	0.99
Current/ex-smoker	13 (72)	13 (76)	0.77
Lowest resting ABPI	0.71 (0.23)	0.72 (0.15)	0.97
Mean (SD) body mass index, kg/m^2	27.0 (4.3)	29.0 (5.6)	0.39
Ischaemic heart disease	6 (33)	8 (47)	0.41
Dyslipidaemia	16 (89)	16 (94)	0.58
Hypertension	14 (78)	15 (88)	0.41
Diabetes mellitus	6 (33)	10 (59)	0.13
Antiplatelet therapy	18 (100)	17 (100)	0.99
Statin therapy	18 (100)	17 (100)	0.99
Anti-hypertensive therapy	14 (78)	15 (88)	0.41

SD: standard deviation; ABPI: ankle brachial pressure index.

Groups were considered together and compared with non-responders from both Groups.

Discussion

This study has demonstrated that a combination of treadmill-training and lower limb resistance exercises may be superior to treadmill-only training with respect to changes in endothelial function. While neither SET regimen had an impact on the non-invasive measures of endothelial function, FMD and RH-PAT, treadmill-only training led to a potentially detrimental reduction in serum NO. In contrast, a potentially beneficial reduction of serum ADMA, the potent endogenous inhibitor of NO, was observed only in the combination group. We were unable to demonstrate a superior effect of the combined resistance and treadmill training on PFWD. We have previously speculated that this may be due to variability in response to SET and the lack of exposure to a sufficient volume of either treadmill or resistance training to enable an adaptive response to either modality to occur.[19] Furthermore, the lack of difference between markers of endothelial function in responders and non-responders to SET challenges the theory that improvement in endothelial function is a mechanism by which SET produces improvement in walking performance.

A reduction in NO bioavailability is a potential detrimental impact of treadmill-based SET on the endothelium that has not previously been reported. It is possible that the repeated low-grade ischaemia-reperfusion injury (IRI) experienced by claudicants during treadmill-based SET may increase the level of oxidative stress to which the endothelium is exposed, thereby damaging the endothelium and reducing NO bioavailability. Reduced exposure to IRI in a combination-based SET resulted in no change to NO level but a reduction in the level of ADMA, an endogenous inhibitor of NO synthesis, was the likely benefit of such a program. While this could be expected to manifest as an improvement in NO bioavailability and endothelial function, this was not the case in our study, perhaps a reflection that ADMA is not the sole antagonist of NO function.[20]

Our findings are in contrast to existing literature in which improvement in endothelial function has been observed following exercise in claudicants; however, a fundamental technical difference is a likely explanation for such conflict in results. Previous studies have demonstrated improvement in FMD using proximal cuff occlusion techniques.[10,21] It has recently been established however, that with such a technique endothelium dependent responses contribute only 40% of the observed dilatation, with significant myogenic and sympathetic responses, both of which are independent of the vascular endothelium.[22] Distal occlusion causes a lesser but more specifically endothelium dependent NO-mediated dilatation and is presently the recommended FMD method[17] and the method chosen for this study.

While there is limited literature for comparison, a correlation between FMD arising from proximal

Table 2. Comparison of baseline, 12 week and absolute change for outcomes between participants in the treadmill only supervised exercise training (Group 1) and the treadmill and resistance exercise training (Group 2). Data presented as median (IQR) unless otherwise stated. For ITT purposes n-values vary according to whether last outcome carried forward was used or where no baseline measure available the follow-up value remained missing.

	Treadmill only (Group 1)				Treadmill and Resistance Training (Group 2)				Between groups
Outcome	Baseline	12 Weeks	Change	*P*-value	Baseline	12 Weeks	Change	*P*-value	*P*-value
Intention to treat									
PFWD, metres[a]	160 (84)	204 (97)	44 (80)	0.03	181 (90)	188 (109)	7 (135)	0.82	0.42
Quality of life[a]	20.4 (3.3)	18.7 (4.2)	−1.6 (2.4)	0.01	22.3 (4.0)	19.0 (4.6)	−3.3 (3.9)	0.01	0.18
Flow-mediated dilatation, %	1.3 (0.7, 3.0)	1.3 (0.9, 4.6)	0.3 (−0.3, 1.9)	0.281	2.4 (0.4, 5.0)	1.2 (0.1, 4.4)	−0.8 (−2.7, 0.8)	0.382	0.110
Reactive Hyperaemia Index[b]	1.7 (1.6, 2.2)	2.1 (1.5, 2.4)	0 (−0.2, 0.5)	0.824	1.6 (1.3, 1.9)	1.6 (1.4, 1.8)	0 (−0.2, 0.3)	0.689	0.769
Nitric Oxide, μmol/L[a]	15.0 (10.3)	8.3 (5.4)	−6.3 (7.4)	0.003	11.2 (5.5)	9.1 (7.4)	−2.1 (5.5)	0.138	0.066
ADMA, μmol/L	0.58 (0.56, 0.67)	0.58 (0.51, 0.65)	0 (−0.07, 0.07)	0.776	0.61 (0.56, 0.67)	0.56 (0.50, 0.63)	−0.03 (−015, 0)	0.028	0.193
Per protocol									
PFWD, metres[a]	170 (82)	221 (97)	50 (78)	0.03	170 (90)	188 (127)	18 (147)	0.67	0.59
Quality of life[a]	19.5 (2.2)	17.6 (2.9)	−1.9 (2.5)	0.01	21.9 (3.6)	19.1 (5.0)	−2.8 (3.7)	0.02	0.45
Flow-mediated dilatation, %	1.4 (1.0, 3.2)	1.4 (0.9, 4.9)	0.6 (−0.3, 2.1)	0.281	2.9 (0.2, 5.0)	1.2 (0.1, 5.0)	−1.0 (−2.9, 0.43)	0.272	0.075
Reactive Hyperaemia Index[b]	1.7 (1.6, 2.1)	2.1 (1.5, 2.3)	0 (−0.3, 0.6)	0.824	1.5 (1.1, 2.2)	1.6 (1.3, 1.7)	0 (−0.3, 0.3)	0.906	0.928
Nitric Oxide, μmol/L[a]	15.4 (10.5)	8.2 (5.6)	−7.2 (7.5)	0.002	9.2 (3.6)	7.5 (7.1)	−1.7 (5.1)	0.282	0.040
ADMA, μmol/L	0.58 (0.56, 0.64)	0.57 (0.51, 0.64)	0.01 (−0.07, 0.08)	0.776	0.65 (0.56, 0.68)	0.56 (0.49, 0.64)	−0.05 (−0.16, 0.01)	0.050	0.236

ADMA: Asymmetric dimethylarginine; PFWD: pain free walking distance.
[a]Data presented as mean (SD).
[b]RH-PAT technology was unavailable for the first 6 participants and therefore collected from only 15 (10M, 5F) and 14 (11M, 3F) participants in Group 1 and 2 respectively.

occlusion techniques and RHI has been reported.[7] Given the improvement in proximal-cuff FMD observed previously in claudicants following SET[10,21] it could be expected that RH-PAT may demonstrate improvement in endothelial function. The lack of change detected in this study may be representative of the fact that there are differences in the response of resistive and conduit vessels to reactive hyperaemia and RH-PAT measures the response in the digits, a vasculature which is a combination of macro and microcirculation with a significant role played by arterio-venous anastomoses primarily regulated by the sympathetic nervous system.[22] Alternatively, the effect size may not have been within the 20% required to overcome the measurement error associated with this technique.[22]

With respect to NO analysis, evidence suggests that metabolites of NO, nitrite and nitrate, used to calculate levels of NO are closely dependent on dietary intake and therefore may not be specific markers for NO degradation.[23] Failure to control for the impact of diet on NO levels is not only a limitation of our study but also of previous studies which in contrast to us, have demonstrated improvement in NO bioavailability following SET in claudicants.[24] A variation in dietary intake may therefore provide some explanation for such contrasting results. Furthermore, Allen et al.[24] measured nitrite levels only which are rapidly metabolised to nitrate and therefore unlikely to provide an accurate representation of total endogenous NO production. The prescribed SET in Arosio et al.[11] was a short (14 day), high-volume program incorporating dynamic exercises such as lunges and squats thus limiting exposure to IRI. Significantly, urinary metabolites of NO measured in Arosio et al.[11] had normalised after 7–10 days. Whether or not a longer duration of the same high frequency and high volume exercise would enable a more sustained beneficial effect on NO bioavailability remains to be seen and is worthy of further exploration.

To summarise, while this study is limited by a small sample size, it provides preliminary evidence to suggest that treadmill-based SET may have a detrimental effect on the vascular endothelium. In the absence of change to the more established measures FMD and RH-PAT and the lack of any long-term clinical follow-up to assess health outcomes in claudicants participating in SET, care must be taken in interpreting such findings. Currently, the literature assessing this area is scant and interpretation of what has been previously reported is complicated by the heterogeneity that exists between studies. Given the well-established link between ED and future cardiovascular-related morbidity and mortality, further research is required to assess the potential detrimental impact of SET on claudicants and consideration should be given to alternative exercise regimens such as the combination-based program utilised in this study.

Conflict of interest

None declared.

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Change in dietary intake of adults with intermittent claudication undergoing a supervised exercise program and compared to matched controls

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Abstract

Background: Presence of numerous diet responsive comorbidities and high atherosclerotic burden among adults with intermittent claudication demands attention is given to diet in an effort to delay progression of peripheral artery disease. The aim of this study was to compare diet of adults with intermittent claudication: (a) against dietary recommendations; (b) following 12 weeks of supervised exercise training; and (c) against non-peripheral artery disease controls.

Methods: Diet was assessed using a food frequency questionnaire pre and post supervised exercise training. Pre-exercise diet was compared against Suggested Dietary Targets and against non-peripheral artery disease controls matched for gender, age and body weight. Pre-exercise diet was also compared against post-exercise diet.

Results: Pre-exercise 25/31 participants, 5/31 participants, 16/31 participants and 4/31 participants achieved recommendations for protein, carbohydrate, total fat and saturated fat respectively. Few achieved recommended intakes for fibre (3/31 participants), cholesterol (8/31 participants), folate (11/31 participants), potassium (1/31 participants), sodium (4/31 participants), retinol equivalents (1/31 participants) and vitamin C (3/31 participants). There were no differences observed between participants compared to controls in achievement of recommendations. Post-exercise, marginally more participants were able to achieve targets for cholesterol, sodium and vitamin C but not for any other nutrients.

Conclusions: Despite evidence to support benefits of dietary modification in risk reduction of peripheral artery disease, adults with intermittent claudication continue to consume poor diets. Research is required to determine whether dietary changes can be achieved with greater attention to nutrition counselling and the impact assessed in terms of delayed disease progression and long term health outcomes.

Trial Registration: ClinicalTrials.gov: NCT01871779.

Keywords: Peripheral arterial disease, Diet, Nutrition, Supervised exercise, Claudication

Background

Peripheral arterial disease (PAD) is a local manifestation of the diverse pathophysiological processes associated with the systemic disease state atherosclerosis. The pro-inflammatory and pro-oxidative nature of the disease together with resultant immobility and impaired quality of life precipitates a nutritional vulnerability in these patients which may compound an already increased risk of coronary and cerebrovascular disease and subsequently premature death [1]. The nutritional vulnerability of these patients takes extra significance when one considers that current international consensus guidelines recommend a supervised exercise program as a treatment for all patients with PAD manifesting as symptoms of intermittent claudication but gives little attention to nutrition education and counselling [2].

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Peripheral arterial disease ordinarily co-exists with numerous diet-responsive cardiovascular and metabolic comorbidities such as hypertension, hyperlipidaemia and diabetes mellitus [3]. It is well documented that dietary modification can delay progression of atherosclerosis [4] and that some nutrients appear to be protective against the development of PAD [5]. For those with PAD, there is increasing epidemiological evidence that single nutrients such as carnitine [6] or multi-nutrient combinations [7] may facilitate improved outcomes. Despite this array of evidence, the dietary intake of adults with PAD has not been extensively investigated.

In the only relevant study Gardner and colleagues [8] compared the dietary intake of 46 males with PAD of varying severity with accepted dietary recommendations and observed that none were able to achieve the recommendations for sodium and vitamin E, and only a small percentage were able to achieve the recommendations for folate (13%), saturated fat (20%), fibre (26%) and cholesterol (39%). The authors concluded that adults with PAD have poor diets. This is despite there likely being several teachable moments during progression of the disease for dietary advice to be provided.

Extensive evidence demonstrates regular exercise is favourable for cardiovascular health and can improve walking performance in patients with IC [9,10]. Unlike diet, exercise is routinely recommended for conservative management of adults with PAD manifesting as IC, appearing as a key recommendation in commonly adopted clinical practice guidelines and systematic reviews [2,11]. From a nutritional perspective, the impact of such an intervention has not yet been established but may be balanced by two contrasting outcomes. Firstly, the increase in metabolic demands associated with exercise [12] may lead to a decline in nutritional status and physical health and further highlights the importance of nutritional status in this vulnerable group of patients. Secondly, given that in other settings, exercise training may improve dietary composition even in the absence of nutrition counselling [13-15] it could be speculated that a spontaneous improvement in dietary intake might occur and provide an additive benefit to exercise therapy for adults with IC. If exercise can result in optimising dietary intakes then benefits might include delayed progression of PAD and improved management of co-existing chronic disease risk factors.

The aim of this study was to compare usual dietary intake of adults with IC: (a) to suggested dietary targets; (b) following 12 weeks of SET; and (c) against non-PAD controls.

Materials and methods

Subjects

22 men and 9 women aged ≥49 years with IC were recruited from the Southern Adelaide Local Health Network Vascular Surgery Claudication Clinic to participate in a 12 week supervised exercise training (SET) intervention designed to maximise walking performance. The SET ran over 12 weeks and consisted of two 60-minute sessions per week supervised by a senior physiotherapist. The SET included treadmill-based training with or without resistance training and is described in detail elsewhere [16]. In addition the participants received counselling on cardiovascular risk factor and lifestyle modification by the medical staff (smoking cessation, regular exercise, healthy diet, weight loss) and attended an educational lecture series, including a 30 minute seminar on healthy diet for PAD. Patients attending the Claudication Clinic with a clinical history consistent with IC, ankle brachial pressure index (ABPI) <0.9 in any ankle artery and radiographic evidence of infra-inguinal disease in the absence of aorto-iliac disease were eligible for inclusion. Those attending the Claudication Clinic who had experienced lower limb ischaemic rest pain, had clinical evidence of tissue loss such as ulcers or necrotic lesions, had undergone arterial intervention (endovascular or open surgery) in the preceding 12 months, suffered from pre-existing cardio-respiratory morbidities limiting exercise capacity, had evidence of aorto-iliac disease or were deemed to be not competent of providing written informed consent were not considered eligible for participation in the study. Ethics was approved by the Southern Adelaide Clinical Research Ethics Committee.

Demographics, medical history and clinical characteristics

Comorbidities, current medications, smoking status and demographic data were recorded from medical records. Walking performance was measured using a six-minute walk test, demonstrated as highly reliable and related to the functional and hemodynamic severity in patients with IC [17]. Pain free walking distance was recorded as the distance, in metres, at which participants first reported claudication pain. Body weight and height were measured to 0.05 kg and 0.1 cm using digital scales and a wall-mounted stadiometer respectively, while wearing light clothing and no shoes.

Dietary intake

Energy and nutrient intake for all participants was assessed using the validated 74-item Dietary Questionnaire for Epidemiology Studies Version 2 (DQES v2) [18] pre and post-SET. Validation of the DQES v2 has demonstrated that it can capture similar nutrient intake data as the more burdensome weighed food records and may be used for estimation of dietary intakes over a relatively short time in clinical intervention trials [19].

The DQES v2 contains 74 food items with 10 frequency response options ranging from 'never' to '3 or more times per day' in addition to photographs of scaled portions for

four foods used for calibration of portion size. There are also questions for calibration purposes on the overall frequency of consumption of selected fruits and vegetables and consumption of other selected foods that do not fit easily into the frequency format. The food composition data used to calculate nutrient intake are derived from *NUTTAB95* [20], with supplementation of other data where necessary [21-24].

The Australian and New Zealand Acceptable Macronutrient Distribution Range and Suggested Dietary Targets (SDT's) for lowering chronic disease risk [25] were selected for the dietary intake of participants to be compared against. For energy, individual estimates of total energy expenditure (TEE) were determined according to the equation of Schofield [26] with adjustment for a physical activity level of 1.4 which is considered as very sedentary according to NHMRC [25]. The ratio of energy intake to TEE <0.79 was applied as a cut-off value to define energy under-reporters. This value is consistent with the lower 95% confidence limit of the ratio of energy intake to TEE, where TEE is measured directly by doubly labelled water [27]. For alcohol, <2 standard drinks (<20 g alcohol) daily reduces the lifetime risk of harm from alcohol-related disease or injury according to the NHMRC and hence this was the recommendation applied for the purpose of this study [28].

The dietary intake of participants was also compared against a gender, age (±5 years) and body weight (±5 kg) matched sample of adults with no history of PAD. The non-PAD control sample were participants of a vascular health screening program who were assessed as not having PAD according to ABPI >0.9 and completed the same DQES v2 as those with IC.

Statistical analyses

All values are reported as mean ± SD and n (%) for continuous and categorical data respectively. To compare energy and nutrient intake of those with IC pre and post SET, and separately nutrient intake of those with IC pre-SET against a gender, age and weight matched sample of adults without PAD, paired samples t-tests were applied. Similar comparisons were made according to the proportion achieving the AMDR, SDTs and alcohol intake recommendations using the chi-square or Fishers exact test of association. Significance was set at $P < 0.05$ and all analyses were conducted using IBM SPSS Statistics Version 20.

Results

Subjects

Participant demographics and clinical characteristics pre-SET are summarised in Table 1. The majority of the sample were male (n = 22, 71%), almost half were smokers (n = 14, 45%) and most experienced established risk factors for PAD including hyperlipidemia (n = 26, 84%) and hypertension (n = 26, 84%). The non-PAD controls (n = 23) had similar BMI (27.2 ± 4.3 kg/m^2) to the participants with PAD, more were smokers (n = 14, 61%), but fewer (n = 9, 39%) had established hyperlipidemia or hypertension (n = 9, 39%).

Table 1 Clinical characteristics of participants with peripheral arterial disease and intermittent claudication pre-commencement of 12 weeks supervised exercise training, n = 31

Variables	
Mean (SD) Age, years	70.7 (10.0)
Gender, n (%) male	22 (71)
Mean (SD) Pain Free Walking Distance, metres	157.9 (93.5)
Current smoking, n (%) yes	4 (12.9)
Diabetes, n (%) yes	10 (32.3)
Hypertension, n (%) yes	26 (83.9)
Dyslipidemia, n (%) yes	26 (83.9)
Mean (SD) Body mass index, kg/m^2	28.0 (4.8)

Dietary intake of participants with IC

The mean (SD) macronutrient and micronutrient intake of participants with IC pre-SET is presented in Table 2. Mean (SD) dietary energy intake was 7837 (3111) kJ/day and 13 (42%) were identifiable as under-reporters of energy intake. While the majority of participants achieved the AMDR for protein (n = 25/31, 81%), there were few participants achieving the AMDR for carbohydrate (n = 5/31, 16%), total fat (n = 16/31, 52%), saturated fat (n = 4/31, 13%), polyunsaturated fat (n = 3/31, 10%) and none achieved the AMDR for monounsaturated fat. Similarly, there were few participants who achieved the SDTs for fibre (n = 3/31, 10%), cholesterol (n = 8/31, 26%), folate (n = 11/31, 36%), potassium (n = 1/31, 3%), sodium (n = 4/31, 13%), retinol equivalents (n = 1/31, 3%), vitamin C (n = 3/31, 10%) and none that achieved the SDT for vitamin E.

Table 2 highlights that when participants with IC (n = 23/31) achieving the AMDRs were compared to non-PAD controls, there was no statistically significant difference observed for protein, carbohydrate, total fat, saturated fat, polyunsaturated fat or monounsaturated fat. Similarly, there was no statistically significant difference in the number of participants with IC able to achieve the SDTs for cholesterol, fibre, folate, potassium, sodium, retinol equivalents, vitamin E or vitamin C compared to non-PAD controls. For energy intake, mean (SD) was 7642 kJ (2256) pre-SET compared to non-PAD controls 8260 kJ (2580), P = 0.287 and there was no significant difference in the prevalence of underreporting energy intake between participants with IC (n = 10/23, 44%) and non-PAD controls (n = 6/23, 26%), P = 0.341.

Table 2 Dietary intakes of participants with peripheral arterial disease and intermittent claudication prior to commencing 12 weeks supervised exercise training (SET) and n (%) meeting Australian recommendations[a] in comparison with gender, age (±5 years) and weight (±5 kg) matched non-PAD controls

	Mean (SD) IC pre-SET (n = 31)	AMDR or SDT[a]	n (%) achieving AMDR or SDT[a]		
			IC pre-SET (n = 23)	Non-PAD controls (n = 23)	P value
Protein, g/day	90 (39)				
% kJ from protein	20 (4)	15-25% TE	18 (78)	18 (78)	1.00
Carbohydrate, g/day	194 (81)				
% kJ from carbohydrate	40 (6)	45- 65% TE	4 (17)	4 (17)	1.00
Total fat, g/day	74 (32)				
% kJ from total fat	37 (5)	20-35% TE	15 (65)	9 (39)	0.179
Saturated fat, g/day	29 (13)				
% kJ from SFA	13 (3)	<10% TE	4 (17)	0 (0)	na
Monounsaturated fat, g/day	27 (12)				
% kJ from MUFA	12 (2)	>20% TE	0 (0)	0 (0)	na
Polyunsaturated fat, g/day	13 (7)				
% kJ from PUFA	6 (2)	8-10% TE	2 (9)	1 (4)	1.00
Cholesterol, mg/day	300 (131)	200	2 (9)	2 (9)	1.00
Sodium, mg/day	2592 (1257)	1600	3 (13)	1 (4)	1.00
Potassium, mg/day	2923 (997)	4700	0 (0)	1 (4)	na
Folate, µg/day	284 (109)	300	8 (36)	7 (32)	0.343
Fibre, g/day	M: 24 (8) F: 21 (8)	M: 38 F:28	2 (9)	2 (9)	1.00
Retinol equivalents, µg/day	M: 918 (311) F: 771 (242)	M: 1500 F: 1220	0 (0)	1 (4)	na
Vitamin E, mg	M: 7 (3) F: 5 (2)	M: 19 F: 14	0 (0)	0 (0)	na
Vitamin C, mg	M: 119 (70) F:120 (39)	M: 220 F: 190	3 (13)	3 (13)	1.00

Abbreviations: *AMDR* Acceptable Macronutrient Distribution Range, *F* female, *M* male, *MUFA* monounsaturated fat, *na* not applicable, *PUFA* polyunsaturated fat, *SDT* Suggested Dietary Target, *SET* Supervised Exercise Training, *SFA* saturated fat.
[a]Recommendations according to the National Health and Medical Research Council Nutrient Reference Values for Australia and New Zealand [25].

Table 3 highlights that the number of participants with IC achieving the AMDRs pre-SET compared to post-SET were not significantly different for protein, carbohydrate, total fat, saturated fat, polyunsaturated fat or monounsaturated fat. For energy intake, mean (SD) was 7676 kJ (3143) pre-SET compared to 7931 kJ (3974) post-SET, P = 0.693. There were no statistically significant differences in the number of participants with IC able to achieve the SDTs pre-SET compared to post-SET for fibre, folate, potassium, retinol equivalents, or vitamin E. Post-SET, there was an increase in the number of participants with IC able to achieve the SDTs for cholesterol (n = 9/29, 31% vs n = 8/29, 28%), sodium (n = 7/29, 24% vs n = 4/29, 14%) and vitamin C (n = 4/29, 14% vs n = 3/29, 10%).

Alcohol

The mean (SD) alcohol intake for participants with IC was 8.6 g (13.1) pre-SET and 8.1 g (13.5) post-SET, P = 0.459. 26/31 (84%) of participants with IC achieved the recommended <2 standard drinks daily pre-SET and for those with post-SET data available, 27/29 (93%) achieved the recommendation, P = 0.025. Of those without PAD, mean (SD) alcohol intake was 8.2 g (10.0) and 18/23 (78%) achieved the recommendation for alcohol intake and this was not statistically significant compared to participants with IC pre-SET (P = 0.545).

Discussion

The findings of this study demonstrate that dietary habits of middle-aged to older adults are poor irrespective of whether or not they are affected by PAD. A lifestyle intervention in the form of a supervised exercise program may be able to facilitate improvement in dietary intake with implementation of formal and targeted nutrition education of a greater magnitude than that provided by the current service.

Consistent with findings from Gardner et al. [8], the majority of participants with IC in the present study failed to achieve the recommended dietary targets across all key macro and micro nutrients with the exception of protein. This translates to excess total fat, cholesterol and sodium intake and sub-optimal fibre and antioxidant intake, the culmination of which is likely to favour the progression of CVD risk factors, endothelial dysfunction

Table 3 Dietary intake of participants with peripheral arterial disease and intermittent claudication pre and post-commencement of 12 weeks supervised exercise training (SET)

	Pre-SET[a]		Post-SET[b]		P-value[b]	P-value[c]
	Mean (SD)	n (%) achieving AMDR or SDT[a]	Mean (SD)	n (%) achieving AMDR or SDT[a]		
Protein, g/day	88 (40)		94 (52)		0.510	
% *kJ from protein*	20 (4)	23 (79)	20 (4)	27 (93)		0.377
Carbohydrate, g/day	189 (81)		189 (82)		0.962	
% *kJ from carbohydrate*	39 (6)	5 (17)	40 (6)	5 (17)		1.00
Total fat, g/day	73 (32)		77 (43)		0.550	
% *kJ from total fat*	34 (5)	16 (55)	34 (3)	16 (55)		1.00
Saturated fat g/day	28 (13)		29 (17)		0.619	
% *kJ from SFA*	13 (3)	4 (14)	13 (3)	4 (14)		1.00
Monounsaturated fat g/day	26 (12)		28 (17)		0.491	
% *kJ from MUFA*	12 (2)	0 (0)	12 (2)	0 (0)		1.00
Polyunsaturated fat g/day	12 (7)		13 (8)		0.606	
% *kJ from PUFA*	6 (2)	3 (10)	6 (2)	4 (14)		0.371
Cholesterol, mg/day	299 (133)	8 (28)	309 (166)	9 (31)	0.740	0.004
Sodium, mg/day	2537 (1263)	4 (14)	2568 (1474)	7 (24)	0.910	0.001
Potassium, mg/day	2818 (944)	1 (3)	2866 (1186)	1 (3)	0.806	1.00
Folate, µg/day	279 (110)	9 (31)	267 (102)	9 (31)	0.549	0.088
Fibre, g/day	M: 24 (8) F: 20 (9)	3 (10)	M: 25 (8) F: 18 (9)	1 (3)	M: 0.590 F: 0.206	0.103
Retinol equivalents, µg/day	M: 896 (301) F: 756 (254)	1 (3)	M: 815 (298) F: 662 (376)	1 (3)	M: 0.275 F: 0.218	1.00
Vitamin E, mg	M: 7 (3) F: 5 (2)	0 (0)	M: 7 (3) F: 5 (3)	0 (0)	M: 0.151 F: 0.325	na
Vitamin C, mg	M: 117 (71) F: 119 (42)	3 (10)	M: 132 (65) F: 107 (45)	4 (14)	M: 0.945 F: 0.521	0.001

Abbreviations: *AMDR* Acceptable Macronutrient Distribution Range, *F* females, *M* males, *MUFA* monounsaturated fat, *na* not applicable, *PUFA* polyunsaturated fat, *SDT* Suggested Dietary Target, *SET* Supervised Exercise Training, *SFA* saturated fat.
[a]Recommendations according to the National Health and Medical Research Council Nutrient Reference Values for Australia and New Zealand – see cut-offs in Table 2 [25].
[b]Mean difference between pre-SET intake and post-SET intake.
[c]Difference in number achieving AMDR or SDT pre-SET and post-SET.

and subsequently the systemic burden of atherosclerosis. Added to this is the inappropriately low consumption of other nutrients such as folate which may precipitate an anaemic state and worsen the symptoms associated with IC.

Unique to this paper, an assessment of alcohol intake was also conducted demonstrating the presence of excessive levels of consumption among some participants with IC pre-SET and a spontaneous reduction culminating in more participants achieving the recommendations post-SET. While not an established risk factor for PAD, the adverse health outcomes of excess alcohol consumption are well documented and according to the World Health Organisation, alcohol is the third most harmful risk factor for chronic disease onset and progression [29].

Surprisingly, the dietary intake data from non-PAD controls demonstrated equally poor dietary intake compared to participants with IC. Given that poor diet is known to impact on CVD risk factors and disease progression, one would expect patients with IC to have a worse diet than non-PAD controls. A possible explanation for the finding in our study is that participants with IC may have had worse dietary intakes before diagnosis and subsequent referral to a specialist service and have therefore already made significant spontaneous changes to bring them in line with non-PAD controls. The timing between diagnosis and dietary assessment however lies outside the scope of this study so we cannot be definitive in this regard. Alternatively, our findings may reinforce the recently established link between genetics and progression to PAD [30]. That is, the genetic make-up of an individual is ultimately responsible for coordinating the extent of interplay between independent risk factors such as dietary intake and hypertension in order to determine extent to which such risk factors are converted to established arterial disease.

Significantly, this study is the first of its kind to assess change in dietary intake in adults with IC following SET and minimal nutrition education. Small improvements were identified in the number of participants achieving

the recommended intake of sodium, cholesterol and vitamin C, however, such improvements were not observed among other nutrients. This seems to be a common trend with dietary change beyond fat, cholesterol and sodium being uncommon, as is evidenced by studies assessing the impact of exercise intervention on dietary intake in other patient groups [13-15]. While the clinical implications of such small changes are unclear, the demonstrated willingness to change dietary intake displayed by these participants as a result of an exercise intervention and minimal nutrition education provides a framework on which, given an injection of sufficient resources, an appropriately credentialed clinician could capitalise and potentially achieve clinically meaningful improvements in outcomes. Better nutrition education may facilitate improvements in the intake of a greater array of relevant nutrients including omega-3 fatty acids, anti-oxidants and fibre. This could therefore be considered as an opportunity to seize the teachable moment with the potential to improve both long and short term health outcomes in this cohort of patients who are already at a high risk of future CVD morbidity and mortality. A targeted intervention in the future to assess such an area may therefore be warranted.

In addition to exploring an array of macro and micro nutrient intake in the present study we also explored total energy intake of participants with IC. While it might be expected that an exercise program would increase total energy requirements and participants would increase energy intake to compensate for these increased demands, this was not demonstrated in the present study. Rather, energy intake remained consistent for participants with IC from pre-SET to post-SET. Interestingly this trend has in fact been reported previously by Speakman and Selman [31] who demonstrated that while exercise training can lead to increases in resting energy expenditure, compensation occurs via reducing body weight and other components of energy expenditure rather than increasing dietary energy intake. It must be highlighted that the present study did identify a high level of underreporting of energy intake and thus this area warrants further investigation. The implications of the underreporting of energy intake in the present study are important to consider in the context of the findings of the present study. Given the present study demonstrated that few participants achieved the AMDR for total fat and saturated fat or the SDTs for cholesterol and sodium, then a higher energy intake than currently reported would likely result in fewer participants having achieved the dietary targets for these critical nutrients. Conversely, if participants truly had a higher energy intake than reported then intake of nutrients including fibre, vitamin E, folate and vitamin C would have been underestimated.

Some additional limitations that warrant consideration include the sample size, method of assessing dietary intake and the lack of capturing data on complementary and alternative medicines containing key nutrients. While the sample size is not dissimilar to the only other similar study in this area [9], ideally a larger study would be conducted to confirm the findings presented in this study. The method of dietary assessment was via a food frequency questionnaire and, although comprehensively validated, food frequency questionnaires have been demonstrated to underestimate dietary intake in some settings. The food frequency questionnaire also does not allow for capturing nutritional supplements, nor did we specifically ask about these and hence, once again, the estimates of some nutrients may be underestimated in the present study. Further research in this area should attend to this deficit in methodology for the purpose of attaining a more accurate determination of nutrient intake that includes both food and supplements.

Conclusions

In conclusion, the findings of the present study raise several questions which can be considered further in future studies. The present study has established that this high risk group with IC has sub-optimal dietary intake of an array of relevant nutrients. Although small, some spontaneous change occurs with SET and minimal nutrition education providing an indication that there may be a willingness to modify dietary behaviours. An opportunity therefore exists to consolidate nutrition services in this area in order to avoid a missed opportunity to achieve significant short and long term health benefits in a nutritionally vulnerable patient group with potentially much to gain.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CD was involved in the conception and design of the study, recruitment of participants, acquisition of data, analysis and interpretation of data and drafting of the manuscript. MM was involved in recruitment of participants, acquisition of data, analysis and interpretation of data and drafting of the manuscript. KD was involved in analysis and interpretation of data and drafting of the manuscript. JIS was involved in conceiving and designing the study, interpretation of data and revising the draft manuscript for important intellectual content. All authors provided final approval for the manuscript to be submitted.

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