# Does a supervised exercise program for claudication have an adverse effect on muscle, endothelial and immune function?

by

**Doctor Simon Vui Vun**

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# PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH

**Peer Reviewed Publications**

1. Vun SV, Miller MD, Delaney CL, Allan RB, Spark JI. The effect of supervised exercise therapy for intermittent claudication on lower limb lean mass. *Journal of Vascular Surgery*. 2016;64(6):1763-1769.
2. Allan RB, Vun SV, Spark JI. A comparison of measures of endothelial function in patients with peripheral arterial disease and age and gender matched controls. *International Journal of Vascular Medicine*. 2016;2016:6.

**Conference Presentations**

1. Vun SV, Miller MD, Delaney CL, Allan RB, Spark JI. Muscle wasting following the treadmill training of patients with intermittent claudication. Royal Australasian College of Surgeons Annual Scientific Congress, Singapore. 9th May 2014.
2. Vun SV, Michael MZ, Allan RB, Spark JI. Endothelial function & microRNA-92a expression in treadmill training of claudicants. Australasian Society of Vascular Surgery Annual Scientific Meeting, Canberra, Australia. 12th October 2014.
3. Vun SV, Michael MZ, Allan RB, Spark JI. Decreased expression pro-atherogenic microRNA-92a following exercise therapy in patients with peripheral artery disease.
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4. Vun SV, Macardle PC, Miller MD, Delaney CL. The short-term effects of treadmill exercise on circulating sub-populations of monocytes in patients with intermittent claudication.

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**Poster Presentations**

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# SUMMARY

Supervised exercise therapy has become increasingly engrained as the first line treatment for patients who suffer from intermittent claudication. This is on the basis of a large body of clinical studies and meta-analysis, demonstrating significant and meaningful gains in walking distance, together with the suboptimal long-term results of medications and invasive treatment.

There is, however, a concern from some authorities, that the exercise may promote injury to physiological systems and potentially lead to adverse effects for patients. One hypothesis suggests that repeated exposure to the ischaemia-reperfusion phenomenon, which is intentionally provoked by exercise therapy, may lead to adverse consequences for muscle, endothelial or immune function; this is the focus of this thesis.

An adverse effect on muscle mass had previously been reported in a study of patients completing treadmill-training and thus, the first focus of this thesis was to determine where in the lower-limb this was actually taking place.

Firstly, an original analysis protocol to quantify lower limb regional muscle mass using DEXA was developed and demonstrated to be reliable on repeated measures. Secondly, this protocol was applied to a group of patients with claudication before and following completion of 12-weeks of supervised exercise therapy. It was hypothesised that a decrease in muscle mass might be observed in the symptomatic and ischaemic muscle as a result of training. Unexpectedly, there was no change in muscle mass in the symptomatic part of the leg, but there was a significant decrease in muscle mass of the thigh. Whilst reassuring that the ischaemia-reperfusion, that exercising patients with claudication are exposed to, does not result in calf muscle atrophy, this finding has generated more questions as to why and how thigh musculature is affected, and whether this is an adverse effect.

Thirdly, this thesis sought to determine if the 12-week supervised exercise program had any effect on endothelial function assessed by a number of means. A number of small non-protein coding RNA molecules, known as microRNAs, have been increasingly implicated in cardiovascular pathophysiology. One microRNA in particular, miR-92a, appears to have a central role in endothelial dysfunction,

vascular inflammation and response to ischaemic injury. microRNA-92a expression, determined by quantitative PCR, was measured from blood, and skeletal muscle biopsies, together with other markers of endothelial function, flow-mediated dilation, and peripheral artery tonometry. Whilst no change was seen in the latter two measures, microRNA-92a levels were lower following 12-weeks of training, suggesting a positive effect of exercise. This study also reports on the levels of an additional measure of endothelial dysfunction, endocan measured by ELISA, which was elevated in patients with claudication compared to healthy controls.

In addressing the question of the effects of supervised exercise on the immune system, inflammatory monocyte subtypes and CD16 expression were assessed by flow cytometry in healthy controls and patients with claudication following 12-weeks of supervised treadmill training. It was observed that patients with claudication have higher numbers of total and inflammatory monocytes, compared to unselected controls, but this was not affected by training. It was, however, observed that monocyte CD16 expression reduced following the exercise training, again possibly suggesting a positive effect of training in the immune system

Finally, it was again observed that treadmill training results in a significant improvement in walking performance in an overground 6-minute walk test.

Thus, the present collection of studies in this thesis, does not suggest there is an adverse effect on calf muscle, endothelial or monocyte function. Whether the changes in thigh muscles are adverse is so far unknown. The long-term effects are as yet unknown, as the possible difference to home- or land-based training, which should be a focused as part of future study.

# DECLARATION

I certify that this thesis does not incorporate without acknowledgment 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.

Dr Simon Vui Vun

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| | |
|---|---|
| 4S | Scandinavian Simvastatin Survival Study |
| 6MWD | 6-minute walking distance |
| 6MWT | 6-minute walking test |
| ABPI | ankle brachial pressure index |
| ACE | angiotensin converting enzyme |
| ACCORD | Action to Control Cardiovascular Risk in Type 2 Diabetes Trial |
| ADP | adenosine diphosphate |
| ACSM | American College of Sports Medicine |
| ADMA | asymmetric dimethyl arginine |
| ADVANCE | Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation Trial |
| ALI | acute leg ischaemia |
| AHA/ACC | American Heart Association/American College of Cardiology |
| AMP | adenosine monophosphate |
| ARA | angiotensin II receptor antagonists |
| ATP | adenosine triphosphate |
| ATT | Antithrombotic Treatment Trialists Collaboration |
| a.u. | arbitrary units |
| BMI | body mass index |
| CAD | coronary artery disease |
| CAM | cellular adhesion molecule |

| | |
|---|---|
| CAPRIE | Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events Trial |
| CD | cluster of differentiation |
| cDNA | complimentary DNA |
| cel | *Caenorhabditis elegans* |
| CETAC | Comparing Exercise Training with Angioplasty for Claudication trial |
| CHARISMA | Clopidogrel and Aspirin versus Aspirin Alone for the Prevention of Atherothrombotic Events Trial |
| C.I. | confidence interval |
| CLEVER | Claudication: Exercise Vs Endoluminal Revascularization Trial |
| CLI | critical limb ischaemia |
| CLIPS | Critical Leg Ischemia Prevention Study |
| COMPASS PAD | Cardiovascular Outcomes for People using Anticoagulation Strategies PAD Trial |
| CoV | coefficient of variation |
| CRF | chronic renal failure |
| CrCl | creatinine clearance |
| CRP | C-reactive protein |
| Ct | cycle threshold |
| CTA | computed tomography angiography |
| CVD | cardiovascular disease |
| DAB | diaminobenzidine |
| DEXA | dual energy x-ray absorptiometry |

| | |
|---|---|
| DICOM | digital imaging and communications in medicine |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| DUS | duplex ultrasound |
| dUTP | deoxyuridine triphosphate |
| EAS | Edinburgh Artery Study |
| ECG | electrocardiogram |
| ED | endothelial dysfunction |
| EDTA | ethylenediaminetetraacetic acid |
| EDRF | endothelial-derived relaxing factor |
| EF | endothelial function |
| EMLA | eutectic mixture of local anaesthetics |
| ESC | European Society of Cardiology |
| ESVS | European Society for Vascular Surgery |
| eGFR | estimated glomerular filtration rate |
| ELISA | enzyme-linked immunosorbent assay |
| ERASE | Endovascular Revascularization and Supervised Exercise for Peripheral Artery Disease and Intermittent Claudication Trial |
| ESWT | extracorporeal shockwave therapy |
| EUCLID | Ticagrelor versus Clopidogrel in Symptomatic Peripheral Artery Disease Trial |
| EXACT | The Exercise versus Angioplasty in Claudication Trial |
| Fc | fragment crystallisable |

| | |
|---|---|
| FACS | fluorescence activated cell sorting |
| FSC | forward scatter |
| eNOS | endothelial nitric oxide |
| ESM-1 | endothelial cell specific molecule-1 |
| eTG | electronic therapeutic guidelines |
| F | female |
| FDA | US food and drug administration |
| FMD | flow mediated dilatation |
| GOALS | Group Oriented Arterial Leg Study |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| GTN | glyceryl trinitrate |
| GWAS | genome-wide association studies |
| HbA1c | haemoglobin A1C |
| HDL | high-density lipoprotein |
| HMG-CoA | 3-hydroxy-3-methyl-glutaryl-coenzyme A |
| HOPE | Heart Outcomes Prevention Evaluation Trial |
| HPS | Heart Protection Study |
| HR | hazard ratio |
| HRP | horseradish-peroxidase |
| hs-CRP | high sensitivity C-Reactive protein |
| IC | intermittent claudication |
| ICC | Intraclass coefficient |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| IHC | immunohistochemistry |

| | |
|---|---|
| IL | interleukin |
| IMP | inosine monophosphate |
| IPO5 | importin-5 |
| IRONIC | Invasive Revascularization or Not in Intermittent Claudication Trial |
| IRI | ischaemia reperfusion injury |
| LDL | low-density lipoprotein |
| LMWH | low-molecular weight heparin |
| LPS | lipopolysaccharide |
| M | male |
| MET | metabolic equivalent |
| MFI | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| MI | myocardial infarction |
| MIMIC | Mild to Moderate Intermittent Claudication Trial |
| mRNA | messenger RNA |
| miR/miRNA/microRNA | micro ribonucleic acid |
| NE | neutrophil elastase |
| NHANES | National Health and Nutrition Examination Survey |
| NICE | National Institute for Health and Clinical Excellence |
| NLR | neutrophil lymphocyte ratio |
| NO | nitric oxide |
| NOS | nitric oxide synthetase |

| | |
|---|---|
| NNT | number needed to treat |
| NS | not significant |
| OBACT | Oslo Balloon Angioplasty versus Conservative Treatment Trial |
| ONTARGET | Telmisartan, Ramipril, or Both in Patients at High Risk for Vascular Events Trial |
| OR | odds ratio |
| oxLDL | oxidised low-density lipoprotein |
| PAD | peripheral arterial disease |
| PAT | peripheral arterial tonometry |
| PARTNERS | Peripheral Arterial Disease Detection, Awareness, and Treatment in Primary Care Study |
| PCSK9 | proprotein convertase subtilisin/kexin type 9 |
| PCR | polymerase chain reaction |
| PFWD | pain free walking distance |
| PFWT | pain free walking time |
| PIR | poverty income ratio |
| qPCR | quantitative PCR |
| RCT | randomized controlled trial |
| REACH | Reduction of Atherothrombosis for Continued Health Study |
| RESTORE | Resveratrol to Improve Outcomes in Older People with PAD Trial |
| RHI | reactive hyperaemia index |
| RH-PAT | reactive hyperaemia peripheral arterial tonometry |

| | |
|---|---|
| RIPC | remote ischaemic preconditioning |
| ROS | reactive oxygen species |
| RNA | ribonucleic acid |
| RNU6B | small nuclear RNA U6B |
| RR | relative risk |
| RT-PCR | reverse transcription polymerase chain reaction |
| SEP | supervised exercise program |
| SFA | superficial femoral artery |
| SMC | smooth muscle cell |
| SMM | skeletal muscle mass |
| SSC | side scatter |
| SVS | Society for Vascular Surgery |
| TASC | Trans-Atlantic Inter-Society Consensus |
| TBS | Tris-buffered saline |
| TdT | terminal deoxynucleotidyl transferase |
| TNF | tumour necrosis factor |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick end labelling |
| VCAM | vascular cellular adhesion molecule |
| VEGF | vascular endothelial growth factor |
| VSMC | vascular smooth muscle cell |
| WCC | white blood count |
| WIQ | walking impairment questionnaire |

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# 1 INTRODUCTION

Peripheral artery disease (PAD) is a medical condition caused by an atherosclerotic occlusion or narrowing of peripheral arteries. This, in turn, causes a restriction of arterial blood flow to tissues and organs, especially of the lower limbs. Both men and women can suffer from PAD, which affects an estimated 200 million people globally (Fowkes *et al.*, 2013). It is therefore a common condition seen frequently by primary healthcare providers, physicians and surgeons. The clinical features can vary from total absence of symptoms through to the experience of constant pain, ulceration of skin, and necrosis of deeper tissues. Ultimately this can result in amputation of the lower limb. Along the spectrum between these two clinical extremes are patients who suffer lower limb pain precipitated by walking, known as intermittent claudication. This milder manifestation of the disease, which is the most common symptomatic presentation, impacts considerably on patient's daily functioning and quality of life. In addition, long-term survival of patients is significantly reduced (Criqui *et al.*, 1992). Five-year mortality rates of patients with PAD are reportedly similar, if not, worse than breast cancer or lymphoma (Criqui, 2001). Therefore the aims of treatment of intermittent claudication are to relieve the incapacity caused by the symptoms and to improve survival (Norgren *et al.*, 2007). Cessation of smoking is, without doubt, the single most important treatment goal, which affects both disease progression and survival (Jonason & Bergström, 1987; Jonason & Ringqvist, 1985). Optimisation of other conditions such as hypertension and dyslipidaemia are useful in reducing risk of cardiovascular complications (LaRosa *et al.*, 2005), but there is little evidence that doing this improves symptoms or disability. Current options for treating the symptoms of intermittent claudication

include physical, medical and invasive therapies, however, the safest and most effective choice is not yet known despite a sustained research effort and technological advancement. Current evidence supports the approach of recommending supervised exercise therapy based on the widely confirmed observation of improvement in walking capacity in the short to medium term (Lane *et al.*, 2013, 2017; Norgren *et al.*, 2007). Some patients will improve considerably, but others will remain unchanged or worsen. A consensus on the factors that determine this has not been reached. In addition, whether such improvements are sustained in the long-term, slow progression of the disease, or translate into improved survival has not yet been well documented in the literature. Separately, the mechanisms that are thought to explain changes in walking capacity are not widely agreed upon. Despite this evidence gap, many advocate exercise therapy asserting that it can reduce cardiovascular risk (Izquierdo-Porrera *et al.*, 2000), and have encouraged its expanded accessibility (Askew *et al.*, 2014; Lane *et al.*, 2013). Others though have previously questioned assumptions and raised concerns about adverse consequences of exercise in the presence of uncorrected deficient blood supply (Tisi *et al.*, 1997; Hickey *et al.*, 1990; Edwards *et al.*, 1994). In particular, the phenomenon of ischaemia-reperfusion injury, which occurs during the cycles of pain of claudication, has previously been proposed to be injurious both to the local muscle and whole individual through effects on vascular, muscle and immune function (Turton *et al.*, 1998; Andreozzi *et al.*, 2007b; Delaney *et al.*, 2015). Opinions concerning this are varied and investigation attempts to determine the validity of these concepts have been relatively limited. Thus, a deeper understanding of the physiologic effects of exercise in the context peripheral artery disease will allow greater

personalisation of treatment options, and improved outcomes, for intermittent claudication.

## 1.1 **The Research Problem**

A complete understanding of a treatment is a prerequisite for any professional who advocates that treatment. This requires deep knowledge of its potential benefits and harms. Unfortunately, much of the research into exercise for claudication has been narrowly focused on improvements in walking capacity whilst other endpoints such as strength, functional outcomes and potential adverse effects have tended to be neglected. This has resulted in a knowledge gap. A deeper investigation into the changes in muscle, vascular and immune function caused by exercise for patients with claudication will provide a better understanding of possible long-term health effects.

## 1.2 General Aims of this Thesis

The overall aim of this thesis is to examine the relationship between muscle, endothelial and immune function in intermittent claudication and the effect that currently recommended treadmill-based supervised exercise has on these functions.

Specifically, this thesis aims:

1. To determine the impact of the currently recommended supervised exercise therapy on regional skeletal muscle mass in the lower limbs.
    - It was hypothesised that the reduction in lower limb skeletal muscle mass in treadmill walking patients with calf claudication, previously reported in the literature, occurs in symptomatic calf muscles.
2. To determine the effect of currently recommended supervised exercise therapy on potential new biomarker microRNAs implicated in endothelial function and limb ischaemia.
    - It is hypothesised that proatherogenic microRNA-92a levels in serum and muscle of patients with claudication would be decreased in patients undergoing the currently recommended supervised exercise therapy.
3. To determine the effect of currently recommended supervised exercise therapy on proportions of activated subtypes of circulating monocyte.
    - It is hypothesised that pro-inflammatory monocyte subpopulations would be decreased in patients undergoing currently recommended supervised exercise therapy.

## 1.3 Format of Investigation

- Chapter 2: provides an in-depth review of the current literature regarding peripheral artery disease and its management will be presented. The aim of this literature review is to discuss what is a currently known and unknown with regard to effect of exercise on patients with intermittent claudication, in order to provide context for the new studies conducted to address the aims of this thesis. The subsequent sections of the thesis are divided into themes related to muscle endothelial and immune function.
- Chapter 3: describes study setting and design, procedures, laboratory and statistical methods to address the research aims and hypotheses.
- Chapter 4: focuses on muscle and examines the effects of an exercise program on regional skeletal muscle mass and walking performance in patients with intermittent claudication. This includes an analysis the reproducibility of customised regional skeletal muscle mass quantification using dual-energy x-ray absorptiometry (DEXA).
- Chapter 5: examines effects of an exercise program on microRNA-92a, which has been suggested as a novel molecular target in treating ischaemic disease.
- Chapter 6: focuses on immune function and a study examines the effects of an exercise program on circulating proportions of monocytes is different states of activation.
- Chapter 7: of the thesis focuses on endothelial function utilising a cross sectional design to investigate whether a novel endothelial protein is useful in identifying endothelial dysfunction amongst patients PAD and controls and whether it correlates with established methods of non-invasive endothelial function assessment.

Finally, a summary of the findings of the study, the conclusions arising from the results of the research and recommendations for future research are discussed (Chapter 8).

Table 1.1. Laboratory techniques performed during the completion of this thesis.

| |
|---|
| 1. Dual energy x-ray absorptiometry and body composition analysis |
| 2. Flow-mediated dilation |
| 3. Peripheral artery tonometry |
| 4. Ultrasound guided percutaneous skeletal muscle biopsy |
| 5. RNA extraction, and real-time reverse transcriptase polymerase chain reaction from serum and muscle samples |
| 6. Enzyme-linked immunosorbent assay |
| 7. Flow cytometry |

# 2 LITERATURE REVIEW

## 2.1 Overview of Peripheral Artery Disease

### 2.1.1 Definitions of Peripheral Artery Disease and Classification

Peripheral artery disease (PAD) is generally defined as stenotic or occlusive disease of arteries excluding those of the coronary or intracranial circulation. It most commonly affects the lower limb arteries, but also affects the upper limb, renal, mesenteric and extracranial circulations. There are other non-atherosclerotic pathological processes that can lead to lower limb arterial insufficiency such as fibromuscular dysplasia, endofibrosis, arterial entrapment syndromes, and complications from aneurysmal disease or thromboembolic disorders (Mintz & Weinberg, 2015). The diagnosis of non-atherosclerotic occlusive arterial conditions should be considered in young patients without traditional risk factors. Atherosclerotic PAD affecting the lower limbs is most commonly classified by the chronicity of the presentation and sub-categorised by clinical stage. Two main categories of PAD are acute limb ischaemia (ALI) and chronic limb ischaemia (CLI). Acute limb ischaemia is defined as sudden decrease in limb perfusion threatening limb viability presenting within two weeks of the acute event (Norgren *et al.*, 2007). Chronic limb ischaemia, which is the main focus of this thesis, is usually present for greater than two weeks, and can be asymptomatic or present with exercise-induced lower limb pain that is relieved by rest (known as intermittent claudication) at the mild end of the spectrum. At the severe end of the spectrum, patients may suffer with ischaemic rest pain and tissue loss. This later sub-category is now termed chronic limb threatening ischaemia (CLTI) in contemporary guidelines (Aboyans *et al.*, 2018). Two widely accepted historical classification systems, Fontaine and Rutherford, have been useful in allowing

consistency in research and reporting of data, thus informing treatment options (Fontaine, Kim & Kieny, 1954; Rutherford *et al.*, 1997) Table 2.1. In general terms, asymptomatic disease and claudication is treated medically, whereas more severe symptoms such as rest pain or tissue loss tend to mandate aggressive efforts to preserve the limb. A comprehensive discussion of the principles of treatment, risk factor modification and long-term outcomes are provided in Chapter 2.

Table 2.1. Fontaine and Rutherford classifications of peripheral artery disease (Fontaine, Kim & Kieny, 1954; Rutherford *et al.*, 1997).

| Fontaine | | Rutherford | | |
|---|---|---|---|---|
| Stage | Clinical | Grade | Category | Clinical |
| I | Asymptomatic | 0 | 0 | Asymptomatic |
| IIa | Mild claudication | I | 1 | Mild claudication |
| IIb | Moderate to severe claudication | I | 2 | Moderate claudication |
| | | | 3 | Severe claudication |
| III | Ischaemic rest pain | II | 4 | Ischaemic rest pain |
| IV | Ulceration or | III | 5 | Minor tissue loss |
| | gangrene | III | 6 | Major tissue loss |

### 2.1.2 **History of Intermittent Claudication**

A number of authors acknowledge that the first pathophysiogical concept of intermittent claudication was introduced by Parisian veterinary surgeon Jean-François Bouley in 1831 (Cheatle, Coleridge-Smith & Scurr, 1991; Bollinger *et al.*, 2000; Condorelli & Brevetti, 2002). It is said that Bouley noticed initially unilateral, then bilateral, reproducible limping and weakness of the hind-limbs of a merchant's horse. After the horse died, post-mortem examination revealed thrombosis of an aneurysmal abdominal aorta and both femoral arteries (Bollinger *et al.*, 2000). A description of the syndrome in humans was made by Brodie in

1846, but it wasn't until some 30 years after Bouley's description that French neurologist Jean-Martin Charcot 1858 defined the condition and coined the term "intermittent claudication", in a patient who had developed iliac artery aneurysm and thrombosis after being shot (Cheatle, Coleridge-Smith & Scurr, 1991; Bollinger *et al.*, 2000; Condorelli & Brevetti, 2002).

The etymology of the term "claudication" derives from the Latin verb *claudicare*, 'to limp' (Oxford English Dictionary, 2011). This is often cited to be in reference the Roman Emperor Tiberius Claudius Caesar Augustus Germanicus (Roman Emperor 41 to 54 AD) who could only walk short distances due to an unknown disease that is presumed to be either childhood polio or cerebral palsy (Levick, 1990). The similarity of the Emperor's name, his disability, and the word *claudicare* is merely a coincidence, since his name was simply passed down from his father's side of the family who descend from the Claudii patrician household of ancient Rome (Hillard, 2012; Armstrong, 2013; Appler, 2017).

Since the initial descriptions of intermittent claudication, theories of the pathophysiology of intermittent claudication evolved to include vasospasm, perfusion steal, and abnormally high blood viscosity (Condorelli & Brevetti, 2002)

The current era of research into the pathophysiology of claudication has shifted from blood and blood vessel focus following the observation that haemodynamic lesions alone do not explain the physical limitations fully (Gardner *et al.*, 1992). Interest in the metabolic alterations of affected muscles led to the concept of a myopathy that is present in the symptomatic limbs of patients with PAD (Pipinos

*et al.*, 2008b, 2008a). Demonstrated myopathic changes observed in the muscles of patients with claudication include impaired mitochondrial oxidisation of carbohydrates, impaired acylcarnitine metabolism, muscle fibre apoptosis, fibre type shifting and polyneuropathy (McGuigan *et al.*, 2001; Weber & Ziegler, 2002; Regensteiner *et al.*, 1993; Brass & Hiatt, 2000; Askew *et al.*, 2005; Pipinos *et al.*, 2003, 2008b). These changes are discussed further in Section 2.3.4. Chronic exposure to cycles of effort-induced ischaemia and reperfusion is receiving increasing interest as a key player in the pathogenesis of PAD manifestations (Pipinos *et al.*, 2008a) and are discussed further in Section 2.3.5.

### 2.1.3 **Diagnosis**

Like other medical conditions the diagnosis of intermittent claudication is based on a history of symptoms and physical examination findings, which support the provisional diagnosis. Confirmation of the diagnosis can be achieved with the addition of objective vascular investigations. Guidelines for the diagnosis and management of PAD are available from specialty societies and provide recommendations along with supporting evidence levels (Norgren *et al.*, 2007; Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018).

#### 2.1.3.1 **History**

The typical history of intermittent claudication is a complaint of lower limb pain, which begins after walking limiting the ability to continue. Patients often describe the pain as cramping, fatigue, burning or an ache, which typically localises to the calf muscles but can also affect the thigh and buttock. Pain relief usually occurs only when walking is halted and normally resolves within 10 minutes (Rose,

1962). Such a pattern of symptoms is present in approximately one third of patients, however other manifestations can include atypical symptoms, which may or may not cause patients to stop walking (McDermott *et al.*, 2001). Lower limb symptoms are, however, very common and other conditions of the back and lower limb can mimic the symptoms of claudication. These mimicking conditions include venous hypertension and neurogenic causes such as spinal canal stenosis, radiculopathies from spinal nerve root compression, and sensory neuropathies from various causes including diabetes (Norgren *et al.*, 2007). In addition, degenerative of the joints conditions such as Baker's cysts or osteoarthritis of the hip, knee and ankle can also masquerade as intermittent claudication (Norgren *et al.*, 2007). Thus, exertional lower limb pain alone is not specific for PAD or intermittent claudication. Differentiating these conditions from intermittent claudication is possible with the addition of physical examination findings and confirmatory objective testing.

Various questionnaires have been used over the decades in and can be used as adjuncts to history taking to quantify the patient's physical limitation in addition to their estimated claudication distance. Examples include the Edinburgh Claudication Questionnaire (Leng & Fowkes, 1992), San Diego Claudication Questionnaire (Criqui *et al.*, 1996) and the Walking Impairment Questionnaire (WIQ) (McDermott *et al.*, 1998). In addition, advancing technology such as global positioning system and wearable devices can assist in accurate quantification of walking distance in patients own environments (Le Faucheur *et al.*, 2008; Gernigon *et al.*, 2015; Normahani *et al.*, 2015). The history should also include inquiry about comorbidities and risk factors since they may add to the diagnostic probability and are opportunities to optimise and manage and improve through secondary prevention measures (Aboyans *et al.*, 2018).

### 2.1.3.2 **Physical Examination**

A complete and thorough examination of the cardiovascular system is essential in the assessment of patients with suspected PAD as other vascular territories can simultaneously be effected and optimal patient care requires management of complete risk as well as limb symptoms (Norgren *et al.*, 2007). Key elements of the physical examination include cardiac and blood pressure assessment and the presence of a palpable abdominal aortic aneurysm. There are a number of findings, which may be found during the examination of patients with intermittent claudication. Pulse examination, including the femoral, popliteal and pedal pulses, may reveal diminished, absent pulses implying compromised arterial circulation should be elicited routinely (Norgren *et al.*, 2007). The location of pulse deficits can help localise arterial lesions, for example, absent pulses from the popliteal artery down suggest a lesion if the superficial femoral artery and/or tibial arteries, whereas absence of femoral pulses implies an aorto-iliac lesion. Pedal pulse deficit does not guarantee the presence of PAD, however, as a proportion of patients will have absent pulses but no objective evidence of PAD (Criqui *et al.*, 1985b). On the other hand, exaggerated prominence of pulses suggests the presence of arterial aneurysmal disease and should prompt further investigation. Other stigmata of arterial disease may include asymmetrical skin temperature and trophic changes of the dermal appendages such as lack of hair and hypertrophied nails, however, these are not specific for arterial disease. Despite these useful physical findings, some patients with intermittent claudication may have a normal examination and full complement of lower limb pulses. Thus, the presence of PAD should be confirmed with non-invasive testing such as the ankle brachial pressure index (ABPI) described below in Section 2.1.3.3 and 2.1.3.4.

### 2.1.3.3 **Ankle Brachial Index Testing**

First described by Winsor in 1950, the measurement of the ankle-brachial pressure index (ABPI) is the simplest method to confirm the presence of occlusive PAD (Winsor, 1950). In its fundamental concept, the ABPI is the ratio of the systolic blood pressure one of the main ankle arteries divided by an estimate of the peak central aortic pressure, usually the systolic blood pressure measured in the brachial artery. Winsor described the use of pneumo-plethysmography to measure pressure (Winsor, 1950), however, this has since been superseded by continuous-wave Doppler assessment of pulses in combination with an occlusive pneumatic pressure cuff recommended by current 2017 European Society of Vascular Surgery/European Society Cardiology (ESVS/ESC) guidelines (Aboyans *et al.*, 2018). In the absence of arterial disease, the peak systolic pressure of lower limbs is normally higher than peak central pressure thought due to the additive effects of reflected pressure waves (Reneman, 2013). Thresholds for normal and PAD vary, but it is generally accepted that the healthy range of ABPI is between 0.9 to 1.39 (Resnick *et al.*, 2004; Aboyans *et al.*, 2012). As the degree and severity of haemodynamic significant lesions increase, the ABPI decreases, such that those with single level disease usually have ABPI greater than 0.5, where as those with multi-level disease have ABPI <0.5. Patients with intermittent claudication typically have resting ABPIs between 0.5 and 0.9, whereas those with critical limb ischaemia have ABPI <0.4 (Hiatt, 2001; Khan, Farooqui & Niazi, 2008). Early studies on the diagnostic validity of ABPI testing have reported sensitivity and specificity as high as 97% and 100% for the detection on angiographically significant stenoses (Ouriel *et al.*, 1982). A recent meta-analysis, however, that pooled four studies and 569 patients reported that an ABPI of <0.9 had a sensitivity of 75% and specificity of 86% when compared to angiography as the

reference standard (Xu *et al.*, 2013). However, this meta-analysis included studies that had poorly defined the recruitment population and one study that used photoplethysmography (Chung *et al.*, 2010) instead of Doppler ultrasound. A more refined meta-analysis, with a specific focus on claudication, was recently reported by Crawford *et al.* in which they reviewed 49 studies, however, only one study passed their strict inclusion criteria (Crawford *et al.*, 2016). The included study by Vega *et al*. reported a 95-97% sensitivity, 56-89% specificity, 91-98% positive predictive value, and 68-86% negative predictive value, depending on whether Doppler or oscillometry was used (Vega *et al.*, 2011). Notwithstanding, the very good diagnostic performance of ABPI compared to angiography, there are some limitations to ABPI testing. It is well known that in some patients the arteries of the ankle are relatively incompressible due to mural calcification which can results in a supra-normal ABPI. This is most commonly due to a pathological finding known as Mönckeberg's (arterio-)sclerosis. In this situation, the rigidity of the artery precludes compression even when the occlusion cuff exceeds intra-arterial pressure. Consequently, in this situation, significant occlusive PAD may or may not be present and cannot be confirmed or excluded by the ABPI alone. An incompressible ABPI which often occurs in poorly controlled diabetics, however, is a marker of increased future risk of all-cause and cardiovascular mortality (Figure 2.1) (Resnick *et al.*, 2004). In addition, some patients may have ABPI >0.9 at rest, but significant occlusive arterial disease that causes low ankle pressures with exercise. In this latter situation, exercise testing can uncover abnormal haemodynamics.

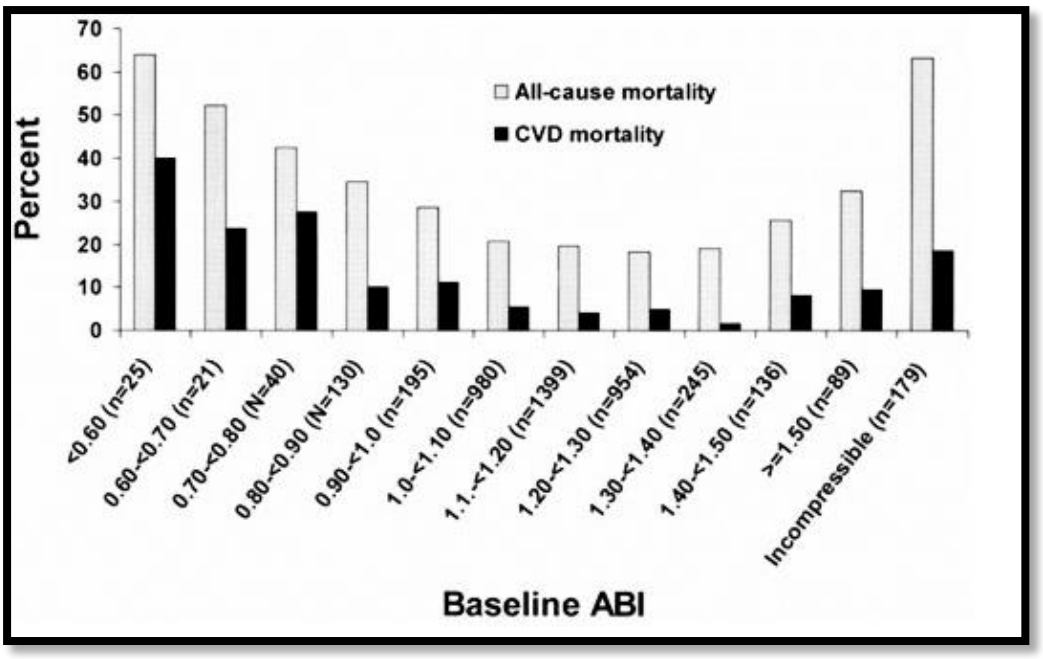


Figure 2.1. The relationship between ABPI and mortality from the Strong Heart Study 1988 to 1999. Image used with permission from Wolters Kluwer Health, Inc. (Resnick *et al.*, 2004)

When coupled with exercise testing, usually with the use of a constant-load or progressive treadmill test, post-exercise ABPIs can be measured. Following exercise, central systolic pressure increases, whilst ankle pressure decreases on average by about 5% due to vasodilation in the active muscle (Ouriel *et al.*, 1982). In the case of significant stenosis, however, the pressure at the ankle decreases precipitously due to increased flow velocity across the lesion which magnifies the loss of energy through kinetic and viscous forces (Kohler, Ted & Sumner, 2013). As a result, post-exercise ABPIs have the ability to detect the presence of a haemodynamically significant pressure gradient that only become apparent during increase flow conditions. This is particularly useful in patients with typical

symptoms of claudication and borderline resting ABPI results (Aboyans *et al.*, 2012).

A number of early studies which investigated the association between ABPI and walking performance reported that there seemed to be no correlation between the two (Szuba *et al.*, 2006; Gardner *et al.*, 1992). However, these studies had significant limitations such as small sample sizes, were confined to treadmill tests, and excluded most patients with PAD due to the absence of classic symptoms of claudication (McDermott *et al.*, 2010). McDermott *et al.,* in a cross-section of n=156 patients with PAD, demonstrated that low ABPI is associated with at least three measures of walking performance, namely 6-minute walk distance, maximal treadmill walking time, and walking impairment questionnaire (WIQ) distance (McDermott *et al.*, 2010). A more recent community study of n=1,566 participants has also demonstrated a correlation between ABPI and longer 400m walk time (McDermott *et al.*, 2013a). Although ABPI has some limitations, there appears to be a significant association between ABPI and walking performance among patients with PAD.

#### 2.1.3.4 **Exercise Testing**

Ankle brachial pressure index testing is often combined with exercise when PAD is suspected, but resting ABPI values are normal. This may occur for instance in the setting of an isolated iliac stenosis or mild disease (Norgren *et al.*, 2007). Current guidelines state that treadmill testing is an excellent tool for objective functional assessment and unmasking moderate disease (Aboyans *et al.*, 2018). Central aortic pressure as measured by brachial pressure increases during and immediately post exercise, whereas vasodilation in exercising leg muscles causes a mild drop in pressure. When this pressure drop is greater than 30mmHg or 20%, it is considered to be diagnostic of 'significant PAD' (Aboyans *et al.*, 2012).

Exercise testing can also quantify walking impairment and has been used since the 1970s. There are two broad categories of exercise testing protocols, namely constant load and progressive/graded (Strandness & Bell, 1964; Hiatt *et al.*, 1988; Gardner *et al.*, 1991). The former rather historical protocol was initially combined with ABPI testing as described above. Despite reasonable reproducibility, certain limitations such as the steep inclination preclude the contact-load test for some frail and elderly patients, and at the other end of the spectrum, patients with mild disease were not able to reach a workload sufficient to provoke symptoms or an increase in pressure gradient (Hiatt, Rogers & Brass, 2014). In contrast, graded treadmill testing allows assessment of walking performance over a wide range of walking abilities beginning at a low-intensity. Graded treadmill tests specific for PAD tend to have a progressive incline, but a constant speed, in contrast to the well-known Modified Bruce protocol which varies incline and speed to progress each stage by approximately 3 METs.

Table 2.2. The most commonly used treadmill exercise testing protocols in PAD.

| Protocol | Type | Incline | Speed |
|---|---|---|---|
| (Strandness & Bell, 1964) | Constant | 12% | 3.2km/h |
| (Gardner *et al.*, 1991) | Graded | Starts at 0%, and increases by 2% every 2 min. | 3.2km/h |
| (Hiatt *et al.*, 1988) | Graded | Starts at 0%, and increases by 3.5% every 3 min. | 3.2km/h |

#### 2.1.3.5 **Imaging**

Imaging studies in PAD can provide information on both structure and function of the arterial system making them useful adjunct to decision making in PAD. Non-invasive imaging includes duplex ultrasonography (DUS), computed tomography angiography (CTA) and magnetic resonance angiography (MRA) whereas catheter angiography is the main modality of invasive imaging. Since most management decisions are based on clinical severity of PAD rather than anatomical severity, vascular imaging has a rather limited role except where surgical or endovascular intervention is contemplated in the setting of severe symptoms or critical limb ischaemia.

### 2.1.4 **Epidemiology**

Global estimates suggest that the overall prevalence of PAD is increasing significantly and is at epidemic proportions. Fowkes *et al*. estimated that over the last 13 years there has been an increase of the prevalence of PAD by about 23.5% meaning approximately 202 million people worldwide are affected (Fowkes *et al.*, 2013). Growth in the prevalence has occurred in both developed and developing countries, however, most of the increase has occurred in low and middle income countries who share almost 70% of the total burden of PAD (Fowkes *et al.*, 2013). On the other hand, high-income countries such as Australia, have seen a 13% increase in the prevalence of PAD (Fowkes *et al.*, 2013). Consequent to the rising overall prevalence, death and disability secondary to PAD has also been rising resulting is more years of life lost and years lived with disability (Sampson *et al.*, 2014).

A number of investigators around the world have measured the prevalence of PAD. In Europe, the Edinburgh Artery Study conducted in 1991, looked at a group (n=1,592) of the general population aged 55-74 and defined PAD as ABPI<0.9 or based on the World Health Organisation (WHO) questionnaire for PAD (Fowkes *et al.*, 1991). Intermittent claudication was present in 4.5% of the sample, but these patients were outnumbered by almost 2:1 by those with significant but asymptomatic PAD. The Rotterdam Study was a cross-sectional population-based study (n=7,715), which showed almost 20% of persons aged >55 years old had PAD (Meijer *et al.*, 1998). The German Epidemiological Trial on Ankle Brachial Index (getABI) Study screened n=6,880 consecutive and unselected patients older than 65 years with ABPI, finding a prevalence of 19.8% of men and 16.8% in women (Diehm *et al.*, 2004). In the USA, an early study of a

geographically defined population (n=613) showed a prevalence of approximately 11.7% in a cohort detected by four different non-invasive methods (Criqui *et al.*, 1985a). More recently, two studies have estimated the prevalence of PAD among communities in the USA. The National Health and Nutrition Examination Survey (NHANES) 2004, a study of n=2,147 men and women aged > 40 years revealed approximately 4.3% had PAD defined by ABPI <0.9. In those aged > 70 years old the prevalence rose to 14.5% (Selvin & Erlinger, 2004). In the Peripheral Arterial Disease Detection, Awareness, and Treatment in Primary Care (PARTNERS) study, Hirsch *et al*. showed prevalence of nearly 30% in patients aged >70 years, or those 50-69 years with a history of either smoking or diabetes mellitus (Hirsch *et al.*, 2001).

#### 2.1.4.1 **Gender Differences in Peripheral Artery Disease**

Historically, it was believed that the prevalence of any PAD is slightly greater in men than in women (Norgren *et al.*, 2007). In the Framingham Heart Study, male gender corresponded to an odds ratio of 1.7 (1.3-2.1, p=0.001) for the presence of PAD compared to women (Murabito *et al.*, 1997). Since women generally live longer than men, however, the absolute burden of PAD was thought to be greater for women, particularly for ages >70 years (Hirsch *et al.*, 2012).

A recent analysis of epidemiologic studies has reported that sex-related differences in the prevalence of PAD and intermittent claudication appear to vary globally and are related to national income data. For example, in high-income countries, as defined by World Bank income groups, there does not appear to be a difference in prevalence between genders (Fowkes *et al.*, 2017). In low-income countries, however, women tend to have a higher prevalence compared to men

when stratified for age. Consistent with this observation is the study by Jensen *et al*. who reported an age-adjusted prevalence of intermittent claudication of 1.1% in men and 1.2% in women (Jensen *et al.*, 2003).

#### 2.1.4.2 **Epidemiology of PAD in Australia**

In Australia, epidemiologic data on PAD is relatively limited. Fowler *et al*. conducted a focused study of men aged 65-83 years in the West Australian capital city of Perth. Using a combination of ABPI and the Edinburgh Claudication Questionnaire the estimated overall prevalence of PAD was 15.6% (Fowler *et al.*, 2002). Later in 2010, Lakshmanan *et al*. investigated the prevalence of PAD amongst 12,203 Perth men aged >65 finding a prevalence of 5.3% (Lakshmanan *et al.*, 2010). Further estimates of the problem can be inferred from epidemiologic studies conducted in other developed nations such as the USA and UK.

At present, there are 11.4 million Australians aged 40 years or older (Australian Bureau of Statistics, 2018b). If one assumes that the prevalence of PAD is similar to that reported by Selvin *et al.* at 4.3% (Selvin & Erlinger, 2004), then the absolute Australian prevalence of PAD can be estimated to be approximately 491,130. The aging Australian population is likely to cause a significant increase in the national burden of PAD (see Section 2.2.1)

Indigenous Australians (Aboriginal and Torres Strait Islanders) unfortunately carry a greater burden of cardiovascular risk and excess morbidity and mortality. In a cohort study of Indigenous Australian in Darwin, after adjusting for other risk factors, the rate of PAD was found to be at least double the rate found in non-Indigenous participants of the AusDiab Study (Maple-Brown *et al.*, 2008; Tapp *et*

*al.*, 2003). Additionally, a recent cohort study of outpatients presenting to a large regional tertiary hospital found that Indigenous Australians make up a small proportion of all patients treated, however, they present at a much younger age, have a higher prevalence of uncontrolled risk factors and suffer poorer clinical outcomes compared to non-Indigenous Australians (Singh *et al.*, 2018).

## 2.2 **Risk Factors**

A risk factor is any attribute, characteristic or exposure of an individual that increases the likelihood of developing a disease or injury (World Health Organization, 2016). The follow section discusses factors which increase the risk of developing intermittent claudication or PAD in general.

### 2.2.1 **Aging**

Age is significant demographic risk factor for PAD. Both the incidence (Dagenais *et al.*, 1991) and prevalence of PAD increase with increasing age (Fowkes *et al.*, 2013). In the Framingham Heart Study, age was shown to be a risk factor with every 10 additional years of age equating to an odds ratio of 1.5 (1.3-1.6, p=0.001) for the presence of PAD (Murabito *et al.*, 1997). This has implications given the age structure of Australia's population is undergoing a significant transformation. Between 1997-2017 there has been a 27% increase in the proportion of people aged 65 years and over, and a 67% increase in the proportion of people aged 85 years (Australian Bureau of Statistics, 2017). Based on this, there are approximately 3.8 million Australians aged 65 years and over in 2017. Furthermore, Australian Treasury data suggests that the number of Australians aged 65 years and over will increase by 148%, from approximately 2.5 million in

2002, to 6.2 million in 2042 (The Treasury, Australian Government, 2017). This will undoubtedly result in a significant increase in the prevalence of PAD and intermittent claudication.

Other risk factors for PAD may be either genetic or non-genetic. Non-genetic risk factors for PAD are predictably similar to those for coronary artery disease given the underlying presence of atherosclerosis and 95% of patients having a least one risk factor of tobacco use, diabetes, hypertension or dyslipidaemia (Selvin & Erlinger, 2004). In addition, these non-genetic risk factors are linked to negative effects on endothelial function described further in Section 2.3.2. Genetic risk factors are discussed in Section 2.2.10.

### 2.2.2 **Smoking**

The link between tobacco use and PAD has been well known for over 100 years. The German neurologist Wilhelm Heinrich Erb was one of the first to publish on the increased prevalence of PAD with increased tobacco exposure, noting that smokers, and heavy smokers were three and six times more likely to suffer intermittent claudication, respectively (Erb, 1911). Further quantification was elucidated in the Framingham Heart study where risk of PAD increased by 1.4 times per 10 cigarettes per day smoked (Murabito *et al.*, 1997). Conen *et al*. demonstrated that cumulative exposure in the form of pack-years increased the hazard ratios significantly among n=39,825 female health care professionals in the USA (Conen *et al.*, 2011). In addition, having a >25 pack-year history of smoking quadruples the risk of developing symptomatic PAD (Price *et al.*, 1999). A high level of smoking also increases the risk of progression to more severe forms of

PAD and increases the risk of death (Jonason & Bergström, 1987). In an Australian study, a legacy effect of smoking was also demonstrated, as shown by Fowler *et al*., where 32% of PAD could be attributed to current smoking and 40% could be attributed to ex-smokers (Fowler *et al.*, 2002). The legacy effect of tobacco use was also observed in the American Women's Health Study (Conen *et al.*, 2011). Promisingly, the rates of tobacco use appear to be decreasing, particularly in younger individuals (Australian Institute of Health and Welfare, 2016). There is currently no data on the risks of vaping or e-cigarette use and PAD despite many anecdotally arguing that it is less harmful. This would be an interesting subject to focus on in future studies in PAD.

### 2.2.3 **Hypertension**

The presence of hypertension is also an independent risk factor for the development of PAD. The Framingham Heart Study demonstrated that men had almost three times, and women almost four times the risk, if they were hypertensive (Kannel & McGee, 1985). In more recent studies, the overall odds ratio for PAD was 1.75 overall in a group of Americans >40 years old (Selvin & Erlinger, 2004). In 2014, there were estimated 4.1 million Australians affected by hypertension (Australian Institute of Health and Welfare, 2016).

### 2.2.4 **Dyslipidaemia**

As with coronary artery disease, dyslipidaemia is also closely linked to the development of PAD. In the Framingham Heart Study, a fasting total cholesterol >7mmol/L doubled the risk of developing PAD. The odds ratio of developing PAD increased by 1.2 for every 1 mmol/L increase in total cholesterol (Murabito

*et al.*, 1997). Ridker *et al*., in the prospective U.S. Physicians' Health study, found that ratio of the total cholesterol to high density lipoprotein the strongest lipid-based predictor for the development of PAD among initially healthy men (highest vs. lowest quartile, RR 3.9, $p<0.001$) (Ridker, Stampfer & Rifai, 2001).

Hypertiglycerideaemia also increased the risk for the development of PAD in the US Physicians Health study (RR 2.8, $p=0.003$). In addition, elevated circulating triglycerides has also have been observed to be predictive of decline in ABPI and progression to critical limb ischaemia (Smith *et al.*, 1996). The combination of smoking and dyslipidaemia appears to be synergistic rather than simply additive (Nakamura *et al.*, 2009).

### 2.2.5 **Diabetes and Glucose Metabolism**

Deranged physiological regulation of glucose metabolism, from impaired glucose tolerance (IGT) to diabetes mellitus, has widely been reported as a risk factor for PAD. In the Framingham Heart Study, diabetics had increased incidence and prevalence of PAD compared to non-diabetics (OR 2.6 (2.0-3.4), $p=0.0001$) for the future development of intermittent claudication (Murabito *et al.*, 1997). Pre-diabetes or IGT is associated with an increased risk of PAD, which appears to be associated with elevated haematological factors (Lee *et al.*, 1999). In diabetics, poorer glycaemic control, as measured by HbA1c, and the duration of diabetes increase the risk of developing PAD (Wattanakit *et al.*, 2005). Similar to other developed nations, diabetes is a significant and growing health problem, with diabetes developing in one Australian every five minutes (Diabetes Australia, 2018). The estimated prevalence of diabetes in Australia was 7.2% in 2015,

corresponding to about 1.7 million people, of which 85% was due to type 2 diabetes (Diabetes Australia, 2018). It is worth noting that not all lower limb complications in diabetics occur in association with large vessel peripheral arterial disease. A significant proportion of diabetic limb complications, such as amputation, occur due to the combined effects of neuropathy, microvascular disease, and impaired resistance to infection. Notwithstanding, PAD in diabetics tends to be diffuse and difficult to treat given its associations with early progression, heavy arterial calcification and crural localisation (Conrad, 1967). In diabetics, medial artery calcification, also known as Mönckeberg's (arterio)sclerosis occurs in apparently healthy media layer in the absence of lipid laden plaques and traditional atheroma (Mönckeberg, 1903; Zazzeroni, Faggioli & Pasquinelli, 2018). This typically occurs in medium and small arteries and is implicated in the development of arterial stiffness (Mackey, Venkitachalam & Sutton-Tyrrell, 2007) and may lead to false elevation of the ABPI (see Section 2.1.3.3). This is considered a distinct process from the intimal calcification that occurs in elastic and medium sized arteries associated with atheromatous plaque development and traditional cardiovascular risk factors.

### 2.2.6 **Chronic Renal Insufficiency**

Chronic renal failure (CRF), diagnosed when glomerular filtration rate (GFR) is less than 60mL/min/1.73m$^2$, or there is objective evidence of kidney damage on urinalysis, present for more than three months (Kidney Health Australia, 2018a). Chronic renal failure is a well-known risk factor for cardiovascular death, coronary artery disease and cerebrovascular disease (Schiffrin, Lipman & Mann, 2007). It is therefore not surprising that patients with CRF also have an elevated

incidence of PAD. O'Hare *et al*. prospectively followed n=2,763 postmenopausal women for up to 8 years, finding that women with creatinine clearance (CrCl) 30-59mL/min/1.73m$^2$ had a significantly increased risk of experiencing PAD events (amputation, revascularisation or lumbar sympathectomy) (HR 1.63; 95% CI, 1.04-2.54, p=0.032) even after correcting for traditional cardiovascular risk factors and medication use (O'Hare *et al.*, 2004). Furthermore, the risk of experiencing PAD events increased further if CrCl decreased to <30ml/min/1.73m2 (HR, 3.24; 95% CI, 1.2-9.78, p=0.021). Similar findings were noted in the NHANES cross-sectional study which demonstrated that adults >40 years old with renal insufficiency (CrCl <60mL/min/1.73m2) had more than twice the odds (OR 2.5, 95% CI 1.2 to 5.1, p= 0.011) of having PAD defined by ABPI <0.9, independent of comorbid conditions and traditional risk factors (O'Hare *et al.*, 2004). Like diabetes, CRF poses a significant problem with about 1.7 million Australians living with reduced kidney function, the risk being approximately tripled for Indigenous Australians (Kidney Health Australia, 2018b).

There are a number of factors unique to CRF that are thought to contribute to the elevated risk of atherosclerotic disease. In renal failure patients traditional cardiovascular risk factors such as hypertension and dyslipidaemia are increased (Drüeke & Massy, 2010). These factors can contribute to endothelial dysfunction and in their own right, however, there are additional factors that are present as a result of CRF that likely explain the accelerated development of atherosclerosis. Patients with CRF have elevated levels of angiotensin II through activation of the renin-angiotensin system (Remuzzi *et al.*, 2005). Angiotensin II stimulates the production of the superoxide radical producing enzyme, nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase, which contributes to oxidative stress

and drives a proinflammatory state by inducing the production of IL-6 and other cytokines (Touyz & Schiffrin, 2004). Superoxide scavenging of NO also contributes to endothelial dysfunction and accelerated atherosclerosis (Vaziri *et al.*, 2002). In addition, endothelial dysfunction is further promoted by asymmetric dimethyl arginine (ADMA) which is a competitive inhibitor of cellular L-arginine uptake and thus NO synthesis (see Section 2.3.2.3). With normal renal function ADMA clearance is performed by the kidney after being metabolised by the renal enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Palm *et al.*, 2007). In CRF, however, circulating ADMA is inversely related to glomerular filtration rate (GFR) and is strongly associated with atherosclerosis and cardiovascular morbidity and mortality (Kielstein *et al.*, 2002). Chronic renal failure also results in the promotion of vascular calcification due to the presence of uraemic serum, increased calcium and phosphate levels, elevated parathyroid hormone. Together with elevated levels of reactive oxygen species (ROS), these factors promote the phenotypic switch of vascular smooth muscle cells into osteoblast-like cells which can produce bone precursors (Vaziri *et al.*, 2003)

### 2.2.7 **Social Disadvantage**

Pande and Creager examined the NHANES cohort of n=6,791 adults >40 years old, looking for associations between markers or poverty and socioeconomic status (Pande & Creager, 2014). Using a ratio of self-reported income relative to the poverty line, termed poverty-income ratio (PIR), the authors found that individuals with the lowest PIR (compared to the highest) had nearly twice the odds of PAD even after adjusting traditional risk factors (OR 1.64, 95% CI 1.04-2.6, $p<0.034$). The highest attained level of education was also associated with increased PAD prevalence but was not significant after multivariable

correction. Among patients with PAD, those with low income or education level are twice as likely to be hospitalised for PAD (Vart *et al.*, 2017). Similar findings of association are found between socioeconomic status and cardiovascular disease in the Australian population (Australian Institute of Health and Welfare, 2017b). These data highlight the excessive burden of PAD carried disproportionally by the socially disadvantaged.

### 2.2.8 **Chronic Inflammation**

Chronic inflammation has been increasing recognised as key player in the development of atherosclerosis and coronary artery disease and many studies have shown that chronic low-grade inflammation increases the risk of cardiovascular events such as stroke and acute coronary syndromes (Ridker *et al.*, 1997; Conen *et al.*, 2009; Ridker *et al.*, 1998; Ridker, Stampfer & Rifai, 2001). The role of inflammatory pathways is discussed in Section 2.3.3.

A prospective cohort study of n=27,935 US female health professionals measured a number of potential biomarkers at baseline and followed them for a median of 12.3 years (Pradhan *et al.*, 2008). Over the study period there were 100 incident cases of PAD and many biomarkers (CRP, fibrinogen, homocysteine, creatinine, lipoproteins) were associated with an increased risk of developing PAD on univariate analysis. However, in a multivariate analysis controlling for traditional risk factors, only hs-CRP (adjusted HR, 2.1; 95% CI, 1.2-3.7) and ICAM-1 (adjusted HR, 4.0; 95% CI, 1.9-8.6) were significant independent predictors (Pradhan *et al.*, 2008). A subsequent analysis of this data was performed to evaluate the relationship between metabolic syndrome, inflammatory biomarkers and development of PAD (Conen *et al.*, 2009). This showed metabolic syndrome was significantly associated with subsequent development of PAD, however, this

association was weakened when biomarkers were added to the model suggesting that increased risk associated with metabolic syndrome is largely mediated through inflammatory pathways and endothelial stimulation (Conen *et al.*, 2009).

Similar associations between markers of inflammation and development of PAD have been observed in the male population. Prospectively collected cohort data, matched for age and smoking habit from the US Physicians Health Study showed that median baseline CRP levels where significantly higher in those who went on to develop PAD compared to those who did not (1.34 vs. 0.99 mg/L, p=0.04) (Ridker *et al.*, 1998). In addition, the risk of developing PAD correlated positively with increasing levels of CRP, and remained significant after controlling for traditional risk factors in multivariable models. Moreover, the risk of experiencing other adverse cardiovascular events is highest among PAD patients who also have a high CRP (>3.0mg/L) (Beckman *et al.*, 2005). Lowering circulating level of CRP is possible through several interventions such as statin medication, dietary manipulation and weight loss, however it is not known whether these translate into improved function level or mortality in patients with PAD.

Interleukins (IL) are inflammatory communication molecules called cytokines that are produced by inflammatory cells and activated endothelial cells. One particular interleukin (IL-6) has received considerable attention as a biomarker in peripheral artery disease. In the cross-sectional Italian InChianti Study, IL-6 was associated with independently associated with PAD cases versus control (McDermott *et al.*, 2005). The prospective Edinburgh Artery Study demonstrated IL-6 to be associated with progressive disease after adjustment for traditional risk factors and novel biomarkers (Tzoulaki *et al.*, 2005). It is worth noting that the IL-6 and CRP are intrinsically linked since the former is the stimulus for the hepatic synthesis of

the later (Khawaja & Kullo, 2009). In addition, IL-6 may be increased by physical activity in patients with PAD (Signorelli *et al.*, 2003) and associated with worse functional capacity (Nylænde *et al.*, 2006). The long-term consequences of this are not known, but are potentially important given the current recommendations for exercise training for patients in intermittent claudication.

### 2.2.9 **Other Risk Factors for PAD**

Other factors such as polycythaemia (high haematocrit) and hyperfibrinogenaemia have been associated with future development of PAD and predict poor outcomes (Lowe *et al.*, 1993; Lane *et al.*, 2006). Both are thought to increase blood viscosity and thus increase coagulation abnormally, and are known to be associated with smoking (Van Tiel *et al.*, 2002; Norgren *et al.*, 2007).

Fibrinogen (Factor I) is the hepatocyte produced circulating glycoprotein precursor of fibrin (Factor Ia). Circulating levels of fibrinogen in contrast to CRP, seem to correlate with severity of lower limb ischaemia as measured by the ABPI (Philipp *et al.*, 1997). Smoking cessation can help to normalise haematocrit and fibrinogen and thus reduce hyperviscosity (Haustein *et al.*, 2002). Fibrinogen levels can also be reduced by exercise (Ernst, 1993) and moderate intake of alcohol (Brien *et al.*, 2011), but is not known whether these effects translate into a reduced disease progression or adverse cardiovascular events and mortality among patients with PAD.

Homocysteine is a non-protein metabolic intermediate formed during the biosynthesis of amino acids methionine and cysteine. The first association between hyperhomocysteinaemia and atherosclerosis was reported in 1969 by

McCully who noted two children with homocystinuria dying with striking atherosclerotic lesions (McCully, 1969). Hyperhomocysteinaemia is a common finding in patients with PAD being found about 30 times more frequently than in the general population (Norgren *et al.*, 2007) and is more common in patients with rest pain than claudication (Spark, Laws & Fitridge, 2003). It is not clear, however, whether hyperhomocysteinaemia is a causal factor in the development of PAD nor whether it modification affects clinical disease progression (Guallar *et al.*, 2006; Andras, Stansby & Hansrani, 1996). There is, however, in vitro evidence that high level of homocysteine impair nitric oxide production and endothelial function (Stamler *et al.*, 1993), and promote oxidative stress (Loscalzo, 1996).

### 2.2.10 **Genetic Risk factors**

It is widely accepted that there is a genetic component to PAD, however, no single gene has been found to found to cause PAD, and it is likely that PAD results from a complex interaction among of hundreds of genes and environmental factors. Current estimates suggest that approximately 50% of the risk of developing PAD is thought to be accounted for by unknown environmental or genetic factors (Leeper, Kullo & Cooke, 2012).

Genome-wide association studies (GWAS) provide a technique to identify genetic variants that increase the risk of developing a disease among thousands of cases and controls (Bush & Moore, 2012). Matsukura *et al.* used GWAS in a study of 785 cases and 3,383 controls, identifying a risk allele on chromosome 13 that was associated with the reduced expression of the gene for nuclear transport protein, importin-5 (IPO5). Importin-5 plays a significant role in reverse cholesterol transport and is thus thought to have anti-atherogenic function (Matsukura *et al.*,

2015). More work is required to better understand the role of genetics in PAD.

### 2.2.11 **Natural History and Long-term Survival**

The results from the cross-sectional Edinburgh Artery Study showed that the prevalence of asymptomatic PAD was 8.0%, whereas the prevalence of intermittent claudication was about half that, at 4.5% (Fowkes *et al.*, 1991). Similar findings were observed in the NHANES report from 1999-2004, where two-thirds of patients over 60 years old were asymptomatic compared to one-third who reported calf pain on walking (Ostchega *et al.*, 2007). Of those that are symptomatic, intermittent claudication or other leg pain is the most common presentation making up 50-90% of presenting cases (Hirsch *et al.*, 2006). In contrast, critical limb ischaemia is far less common, making up about 1-3% of cases of PAD (Hirsch *et al.*, 2006).

The risk of progression in patients with intermittent claudication has previously been reported to be low, with 10-year risk of major amputation <10% (Muluk *et al.*, 2001). A more recent prospective cohort study of 1,107 patients with de novo intermittent claudication, demonstrated an even lower rate of progression to CLI among patients with claudication of 1.1%, and for major amputation 0.21% at 5-years (Kumakura *et al.*, 2017). Similarly, a recent Swedish study reported a 0.4% per year incidence of amputation among patients with claudication undergoing revascularisation (Baubeta Fridh *et al.*, 2017). Asymptomatic disease is should not be considered “mild”, however, since its presence indicates quintupling of risk of dying from cardiovascular disease compared to patients without PAD (Criqui *et al.*, 1992). Indeed, patients with PAD have a similar risk of death at 5-years as patients with stage 3 colorectal cancer (31%) (Dormandy, Heeck & Vig, 1999; Australian Institute of Health and Welfare, 2017a).

In contrast to the low-risk to the limb, historical data suggests, patients with claudication have a substantial mortality of approximately 12% per year, highlighting the importance of risk reduction therapy in overall clinical management (Muluk *et al.*, 2001).

Long-term survival appears to be related to the haemodynamic severity of disease, with the mortality risk increasing as ABPI falls below 1.0 (Figure 2.3). Additionally, when the ABPI is supranormal, an indication of arterial calcification, the mortality also increased, resulting in a U-shaped relationship between mortality and ABPI (Figure 2.1) (Resnick *et al.*, 2004).

Global data from the Reduction of Atherothrombosis for Continued Health (REACH) registry reported that patients with PAD have the highest risk of cardiovascular events and hospitalisation compared to patients with CAD or cerebrovascular disease (Bhatt *et al.*, 2006b). Indeed, a prospective case-control trial of n=255 males with intermittent claudication followed for 7 years, has shown that the rate of cardiovascular events among patients with claudication has not markedly decreased over the last 20 years (Rantner *et al.*, 2017). However, the advancement in the medical management of risk factors may be starting to improve mortality in these patients with claudication, as the overall mortality rate in this study was 16.1%, with most due to cancer and only 5.1% due to vascular causes.

## 2.3 **Pathophysiology**

### 2.3.1 **Peripheral Ischaemia**

In most cases, the primary pathological process behind the development of PAD, is the development of stenosis or occlusion in one or multiple arteries in supplying the lower limb resulting in relative arterial insufficiency. Such vascular lesions may become physiologically and clinically important depending on other circumstances, such as muscular work, mobility and sensory neuropathy. Atherosclerosis and the development of steno-occlusive plaques is by far the most common reason to develop such lesions in the arterial tree.

Constriction in luminal diameter is of paramount importance given that flow is proportional to the luminal radius to the fourth power as per the Hagen-Poiseuille equation (Figure 2.2)(Pfitzner, 1976). It is important to note however, that early in the pathological process, mild stenoses may not result in haemodynamic insufficiency due to compensatory distal vasodilation that maintains an adequate pressure differential and thus flow to distal tissues. It is not until the atheromatous plaques reduce distal blood flow beyond a level which can be compensated by a reduction in distal resistance to flow do atherosclerotic lesions become symptomatic. Typically, this occurs at 50-75% stenosis or when there are serial stenotic lesions <50% at multiple levels (May *et al.*, 1963).

$$Q = \frac{\pi \mathrm{Pr}^4}{8\eta l}$$

Figure 2.2. The Hagen–Poiseuille equation where $Q$ is the flow rate, P is the pressure difference, r is the radius of the conduit, η is the viscosity, and $l$ is the conduit length.

When this occurs, a supply-demand mismatch can occur resulting in ischaemic tissues. Such a mismatch may be situational, for example, when walking, as working muscles require increased supply of oxygenated blood and removal or accumulated waste products of metabolism such as carbon dioxide and lactic acid. This is the mechanism by which arterial insufficiency is thought to cause symptomatic intermittent claudication. The inability to increase muscular perfusion in the setting of increased muscular work can be quantified objectively by measuring a drop in systolic ankle pressure (see Section 2.1.3.3). This can also be demonstrated on contrast-enhanced perfusion ultrasonography (Kundi *et al.*, 2017). The level of the arterial obstruction is generally reflective of the localisation of symptoms. For example, aortoiliac disease often results in thigh, buttock and calf claudication, whereas infrainguinal disease generally results in calf claudication (Bonney, 1956; Widmer, Greensher & Kannel, 1964). It is worth noting that typical claudication is only manifest in approximately 10-35% of patients presenting with PAD, with 20-50% being asymptomatic, and 30-40% having other leg pain (Norgren *et al.*, 2007). It is unclear what pathophysiologic processes are at play in producing atypical claudication symptoms, but it is likely that environmental factors and comorbid conditions, such as neuropathy, play a

role (McDermott *et al.*, 2001). Regardless of the symptoms, recurrent exposure to ischaemia followed by reperfusion is thought to be important in the development of the adaptations found in patients with claudication, and possibly their modification by different therapies (discussed further in Section 2.3.5).

### 2.3.2 **Role of the Endothelium**

The human endothelium is estimated to consist of approximately ten trillion cells, with a surface area equivalent to six tennis courts, and is thus a huge organ with a mass comparable to five normal hearts (Henderson, 1991; Galley & Webster, 2004). The endothelium, however, is far from being an inert barrier. Furchgott and Zawadzki were first to demonstrate the importance of the endothelium in mediating vasodilatory responses to acetylcholine, noting that if the endothelium was physically removed, acetylcholine would cause dose-dependent paradoxical vasoconstriction (Furchgott & Zawadzki, 1980). They hypothesised in their seminal paper that acetylcholine acting on muscarinic receptors stimulated the release of an endothelial-derived relaxing factor (EDRF) that caused the relaxation of vascular smooth muscle cells. Seven years later, two independent groups demonstrated that the EDRF was indistinguishable from the lipophilic gaseous free radical nitric oxide (NO) paving the way for extensive amount of research on this molecule (Ignarro *et al.*, 1987; Palmer, Ferrige & Moncada, 1987). As a paracrine molecule, nitric oxide has been shown to be critical to the normal function of the endothelium, which includes modulation of cellular adhesion and inflammation, coagulation, vascular tone and angiogenesis (Ignarro, 1989; De Caterina *et al.*, 1995; Lloyd, Yang & Terjung, 2001; Vita *et al.*, 2008; Davignon & Ganz, 2004). In 1998 the Nobel Prize in Physiology or Medicine was awarded to American pharmacologists Furchgott, Ignarro and Murad for the

discovery of NO signalling in the cardiovascular system (SoRelle, 1998; Raju, 2000).

#### 2.3.2.1 **Nitric Oxide Synthase**

Nitric oxide can be synthesised by one of three isoforms of nitric oxide synthase (NOS). In neural tissue and skeletal muscles, neuronal nitric oxide synthase (nNOS) functions to produce NO involved in synaptic transmission, central regulation of blood pressure, neuromodulation of behaviour, plasticity, and memory formation (Schuman & Madison, 1991; Kendrick *et al.*, 1997; Zhou & Zhu, 2009). In inflammatory cells such as phagocytes, an inducible form of NOS (iNOS) functions to support host immunity by producing large amounts of NO in response to proinflammatory cytokines such as interleukin-1 (IL-1), TNF-α and IFN-γ (Macnaul & Hutchinson, 1993; Green *et al.*, 1994). It is endothelial NOS (eNOS), however, that is the most significant in cardiovascular and PAD. Endothelial NOS is a membrane-bound oxido-reductase enzyme consisting of homodimer of two protein monomers, each with a reductase domain at one end and an oxidase domain at the other. Each monomer is encoded for by the NOS3 gene on the long arm of human chromosome 7 (Marsden *et al.*, 1992). A total of four cofactors, reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH4), together with haeme and calcium-bound calcium-modulated protein (calmodulin), in the presence of the amino acid substrate L-arginine is required for the synthesis of NO (Alderton, Cooper & Knowles, 2001). The net reaction catalysed by eNOS and its cofactors is shown in Figure 2.3. Failure of NOS monomers to dimerise due to subnormal levels of BH4 can lead to the production of free radical superoxide rather than NO (Vásquez-Vivar *et al.*,

1998). This can further inhibit NOS dimerization even greater production of superoxide in a positive feedback loop contributes oxidative stress and reduced NO bioavailability in a process known as eNOS uncoupling (Luo *et al.*, 2014).

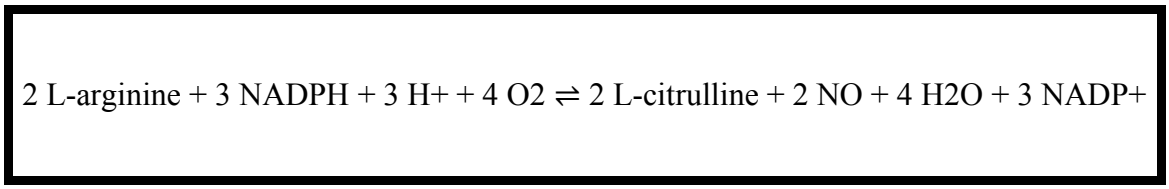


Figure 2.3. The chemical reaction catalysed by eNOS (Knowles & Moncada, 1994).

#### 2.3.2.2 **Effectors of Nitric Oxide**

Once synthesised, NO has a very short half-life, of at most a few seconds (Hakim *et al.*, 1996). Due to its lipid solubility, however, it freely diffuses across the cellular membranes to vascular smooth muscle cells (VSMCs) where it upregulates the production of the second messenger molecule, cyclic guanosine monophosphate (cGMP) by enzyme soluble guanylate catalyse (Förstermann *et al.*, 1986). Cyclic guanosine monophosphate, then activates protein kinase G (PKG) which in turn phosphorylates potassium ion channels (opening them) and L-type calcium ion channels resulting in the closure of calcium-dependent potassium channels (Furchgott & Vanhoutte, 1989). The efflux of potassium ions from the cell augments the closure of voltage-dependent calcium channels and results in reduced influx of calcium ions, which are required for smooth muscle contraction. The end result of NO signalling in this sense is vasodilation and control of vasomotor tone (Vanhoutte *et al.*, 2016).

An important stimulus of NO production, is laminar shear-stress, which imparts a viscous drag force on the endothelium resulting in mechanical deformation of the membrane bound ion channels and cell's ultrastructure (Olesen, Claphamt & Davies, 1988; Cooke *et al.*, 1991). One of the main mechano-sensing structures of the endothelium is the endothelial glycocalyx composed primarily of molecules of heparan-sulphate proteoglycan (Florian *et al.*, 2003). This subsequently leads to signal transduction by various second messenger systems within the cell leading to activation of various kinases such as, protein kinase A (PKA) and B (PKB), and calcium-calmodulin dependent kinase II (Dimmeler *et al.*, 1999). These kinases can then phosphorylate the eNOS protein at various residues, which improves it's calcium sensitivity and it's synthetic efficiency (Fulton *et al.*, 1999; Fleming *et*

*al.*, 2001). Other stimuli, such as VEGF, bradykinin, and oestrogen can also act via their respective receptors to activate PKA and protein kinase C (PKC) and also increase NO production by eNOS (Papapetropoulos *et al.*, 1997; Hisamoto *et al.*, 2001).

In addition, chronic exposure to laminar shear-stress causes increased eNOS activity through increased expression and stability through increased eNOS mRNA synthesis or post-transcriptional modification via polyadenylation (Davis *et al.*, 2001; Weber *et al.*, 2005). Other post-transcriptional regulatory molecules, such as microRNAs, which can also affect eNOS and NO synthesis (discussed further in Section 2.3.3.4.2 on miRNAs). Whether miRNA pathways can be manipulated by exercise interventions has yet to be investigated.

Nitric oxide also has other functions, and can inhibit endothelial adhesion with platelets and leucocytes, inhibit VSMC motility and proliferation. In platelets, NO also activates soluble guanylate catalyse and increases the production of cGMP, which in turn causes platelet inhibition by reducing cytosolic calcium, platelet-selectin (P-selectin) expression, fibrinogen binding to glycoprotein IIb/IIIa receptors, and thromboxane $A_2$ expression (Gries *et al.*, 1998; Wang *et al.*, 1998). The adhesion of leukocytes to endothelial cells is also inhibited by NO through the decreased surface expression and adhesive capacity of adhesion molecules such as CD11 and CD18 leucocyte integrins and intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Kubes, Suzuki & Granger, 1991; De Caterina *et al.*, 1995). These NO regulated processes are thus critical in vascular inflammation and thrombosis and likely contribute to the disease progression of atherosclerosis (Liu & Huang, 2008).

The regulation of endothelial NO and eNOS is summarised in Figure 2.4.

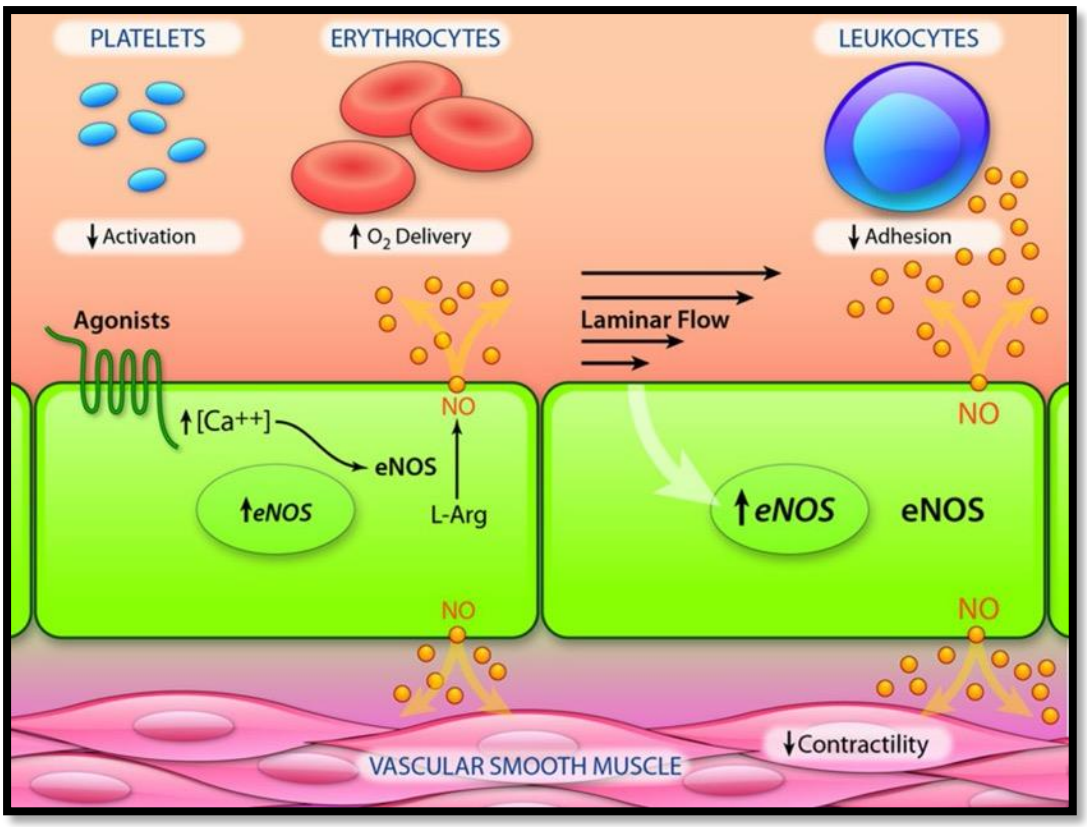


Figure 2.4. The regulation of NO and eNOS production by endothelial cells and its effectors. Image used with permission from Wolters Kluwer Health, Inc. (Gimbrone & García-Cardeña, 2016).

### 2.3.2.3 **Asymmetric Dimethylarginine**

Countering the production of NO by eNOS is the naturally occurring endogenous inhibitor asymmetric dimethylarginine (ADMA) first described by Vallance et al. in 1992 (Vallance *et al.*, 1992). Asymmetric dimethylarginine is synthesised by the methylation of proteins containing the arginine amino acid residues by enzymes called protein arginine methyltransferases (Rawal *et al.*, 1995). Once proteolysis occurs, ADMA is formed and enters extracellular space it enters the circulation and is metabolised by the renal enzyme DDAH and excreted via the

kidney (see Section 2.2.6). Asymmetric dimethylarginine that is not excreted, however, is structurally analogous to L-arginine and can competitively inhibit eNOS, and thus NO synthesis, leading to endothelial function. Both renal dysfunction and oxidative stress have been associated with increased levels of ADMA and decreased NO and endothelial function, thus providing a link between these risk factors and the development of atherosclerosis (Miyazaki *et al.*, 1999; Cooke, 2000). Indeed, plasma ADMA levels correlate positively with PAD severity, and negatively with ABPI and walking time, and independently predict major adverse cardiac events (Wilson *et al.*, 2010). Statin medications, in particular hydrophilic statins such as rosuvastatin, pravastatin and fluvastatin appear to significantly reduce plasma ADMA levels (Serban *et al.*, 2015). However, the endothelial effects of simvastatin and pravastatin appear to be damped by elevated levels of ADMA (Janatuinen *et al.*, 2003; Hsu *et al.*, 2016). Exercise, on the other hand appears to beneficially lower ADMA based on the observation that 12-weeks of regular aerobic exercise decreased plasma ADMA levels in postmenopausal women (Tanahashi *et al.*, 2014). In patients with claudication 12-weeks of treadmill-based SEP failed to reduce serum ADMA, however, combination resistance and treadmill SEP did result in a small but significant reduction (Delaney *et al.*, 2015). An earlier study, however, did demonstrate that 6 months of treadmill SEP resulted in a significant reduction in plasma ADMA (Schlager *et al.*, 2011). The difference in ADMA outcome between these two studies may be explained by the fact that those in latter study were more likely to have severe claudication compared to those in the former. Exercise may therefore improve the effectiveness of statins by lowering ADMA levels, and combined together may offer a synergism with an effect of endothelial function greater than the sum of either alone.

#### 2.3.2.4 **Endothelin**

The vasodilatory effects of NO are counter-regulated by a further antagonist molecule, known as endothelin-1 (ET-1), which is central to the regulation of basal vascular tone (Haynes & Webb, 1994). Endothelin-1 is the most potent endogenous vasoconstrictor currently known and was first defined in 1988 by Yanagisawa et al. (Yanagisawa *et al.*, 1988). Endothelin-1 belongs to a larger family of peptide signalling molecules and ET-1 is synthesised by endothelial cells in response to a range of factors such as catecholamines, angiotensin II, antidiuretic hormone, cytokines and ROS (Kohno *et al.*, 1989; Prasad, 1991; Kanse *et al.*, 1991). Conversely, shear-stress and NO down-regulate ET-1 mRNA (Sharefkin *et al.*, 1991; Malek & Izumo, 1992; Kuchan & Frangos, 1993). Once synthesised, ET-1 activates G-protein coupled ET receptors on the surface of VSMCs resulting, via second messengers, in an increase intracellular calcium concentration and thus vasoconstriction (Neylon, 1999). Of note is that plasma ET-1 levels appear to correlate with Fontaine clinical stage of PAD (Mangiafico *et al.*, 1999). In addition, patients with claudication have been observed to have ET-1 levels are elevated compared to healthy controls, and furthermore, treadmill-based exercise walking exercises caused a doubling of plasma ET-1 levels, whereas controls exhibit no change in levels (Mangiafico *et al.*, 2000). This may in fact signify a potentially harmful effect of exercise in patients with claudication.

#### 2.3.2.5 **Eicosanoid Mediators**

Another important group of circulatory paracrine signalling molecules is the eicosanoids. These molecules are synthesised from membrane phospholipids into

the intermediate molecule arachidonic acid (AA) by either phospholipase $A_2$ or phospholipase C (Ricciotti & FitzGerald, 2011). Arachidonic acid then undergoes oxidation catalysed by one of the prostaglandin-endoperoxide synthase enzymes, commonly known as cyclooxygenases (COX), which are found in the endothelium and other tissues. Cyclooxygenase-1 is the key enzyme involved in the synthesis of endothelial prostaglandins, which is constitutively expressed and primarily responsible for the production of prostacyclin ($PGI_2$). Prostacyclin affects vasomotor tone by activating G protein-coupled receptors on VSMCs, which in turn elevates intracellular levels of cAMP resulting in activation of PKA. Protein kinase A then inhibits the smooth muscle contraction by phosphorylation of myosin light chain kinase (Breyer *et al.*, 2002). Thromboxane $A_2$, however, is an eicosanoid mediator produced by circulating platelets containing COX-1. Thromboxane $A_2$ has the opposite effect of $PGI_2$ on vasomotor tone, that through the activation of G protein coupled receptors, culminates in the increase in cytosolic calcium concentration and thus vasoconstriction (Smith, Araki & Lefer, 1980). In addition, both $PGI_2$ and $TXA_2$ have effects on haemostasis and thrombosis with the former potently inhibiting platelet aggregation, and the latter activating platelets (Smith, Araki & Lefer, 1980).

Aspirin is effective as an antiplatelet agent because it irreversibly blocks the platelet production of $TXA_2$ by COX-1 (see Section 2.4.1.2). Because platelets lack a nucleus and the cellular machinery to synthesise more COX-1, the antiplatelet effect lasts the lifetime of the platelet, usually 8-9 days. In contrast, endothelial cells and other tissues have the machinery to manufacture more COX enzymes thus leaving prostaglandin synthesis relatively preserved.

#### 2.3.2.6 **Endothelial Regulation of Haemostasis**

In addition regulating factors of platelet function described above, the endothelium is key in maintaining the liquidity of circulating blood in direct contact with it by inhibiting key components of the coagulation cascade. A number of membrane expressed proteins and proteoglycans are known to exist that function to prevent inappropriate thrombosis. Firstly, endothelial cells express heparan-sulphate proteoglycans on the luminal surface which serve as a cofactor for antithrombin III (Weitz, 2003). Antithrombin III inactivates the coagulation factors involved in the contact activation pathway, including the serine protease thrombin (Pike *et al.*, 2005). Secondly, the endothelium also expresses tissue factor pathway inhibitor (TFPI), which inhibits factors Xa and VIIa in the coagulation cascade (Wood *et al.*, 2014). Thirdly, thrombomodulin is an integral membrane bound protein which removes the pro-coagulant activity of circulating thrombin (Sadler, 1997). Additionally, the thrombin-thrombomodulin complex activates protein C, which along with activated protein S inactivates activated factors V and VII.

The healthy endothelium maintains an appropriate balance between haemostatic and antithrombotic mechanisms. When endothelial function is disturbed, however, the balance is tipped towards a pro-coagulant, pro thrombotic environment, which can result in disease procession and other cardiovascular complications. Tipping this balance away from thrombosis is a key aim of pharmacological intervention. The effect of aspirin and other antiplatelets is discussed in section 2.4.1.2 and anticoagulants in section 2.4.1.4. Non-pharmacological interventions, such as exercise, may also tip this balance by increasing vascular shear-stress.

Endothelial expression of heparan-sulphate upregulated by shear-stress conditions during in cell culture studies of human endothelial cells (Giantsos-Adams *et al.*, 2013). Thus exercise in patients with claudication may exert some positive effect by favouring heparan-sulphate preservation. Interestingly, nutritional supplementation with exogenous heparan-sulphate has been shown to exert some positive effects, such as improved PFWD, in patients with claudication (Strano, Pinto & Galati, 1990; Messa & Gelso, 2002). Little is known about definite mechanism at play, however, it is proposed to augment the antithrombotic effect of antithrombin III and reduced platelet aggregability (Strano, Pinto & Galati, 1990). The biological effects of exercise therapy on vascular function are discussed further in section 2.4.3.4.

#### 2.3.2.7 **Endothelial Regulation of Inflammation**

One of the other important regulatory functions of the endothelium is that of inflammation. The endothelium occupies a key position between the circulating blood, which contains inflammatory cells and humoral mediators, and the various tissues on the abluminal side. At this interface then endothelial cells effect vascular permeability and adhesion of inflammatory cells. A number of factors can stimulate endothelial cells to allow these functions, and once this process has begun the endothelium can be considered to be activated in a proinflammatory and prothrombotic state, which is now recognised to occur early in the process of atherogenesis (Hunt & Jurd, 1998).

#### 2.3.2.8 **Endothelial Cell Activation and Inflammation**

Endothelial activation is said to occur when a phenotype change in endothelial cells results in the recruitment and trafficking of cells of the immune system.

Whilst this process is generally protective in the case of infection, endothelial cells can be activated by biochemical toxins and biomechanical factors that also lead to inflammation such as oxidised LDL, substances in tobacco smoke, disturbed flow and hypertension (Gimbrone & García-Cardeña, 2016). It is no coincidence that these factors risk factors for atherosclerosis. Once activated endothelial cells mobilise preformed Wiebel-Palade granules containing P-selectin and von Willebrand factor. P-selectin is a cellular adhesion molecule which provides a receptor for leukocyte expressed ligands such as P-selectin glycoprotein ligand-1(Langer & Chavakis, 2009). This interaction allows for the recruitment of leucocytes to activated endothelial cells and sites of tissue inflammation through a minimal affinity binding. Locally produced cytokines by tissue macrophages activate the recruited leukocytes and local endothelial cells to express another form of cell membrane receptors called integrins which permit higher affinity binding than that of selectins and allows the for the immobilisation of leucocytes at sites of endothelial activation and inflammation. Simultaneously, the cytoskeleton of endothelial cells undergoes contraction opening spaces in the normally continuous endothelial barrier. This increases the permeability of the interface and allowing the transmigration of leukocytes and diffusion of humoral factors into the interstitial space. This acute response, known as type I activation, is described as being rapid in onset due to the release of preformed mediators and generally self-limited and reversible (Bach *et al.*, 1995). See Figure 2.5.

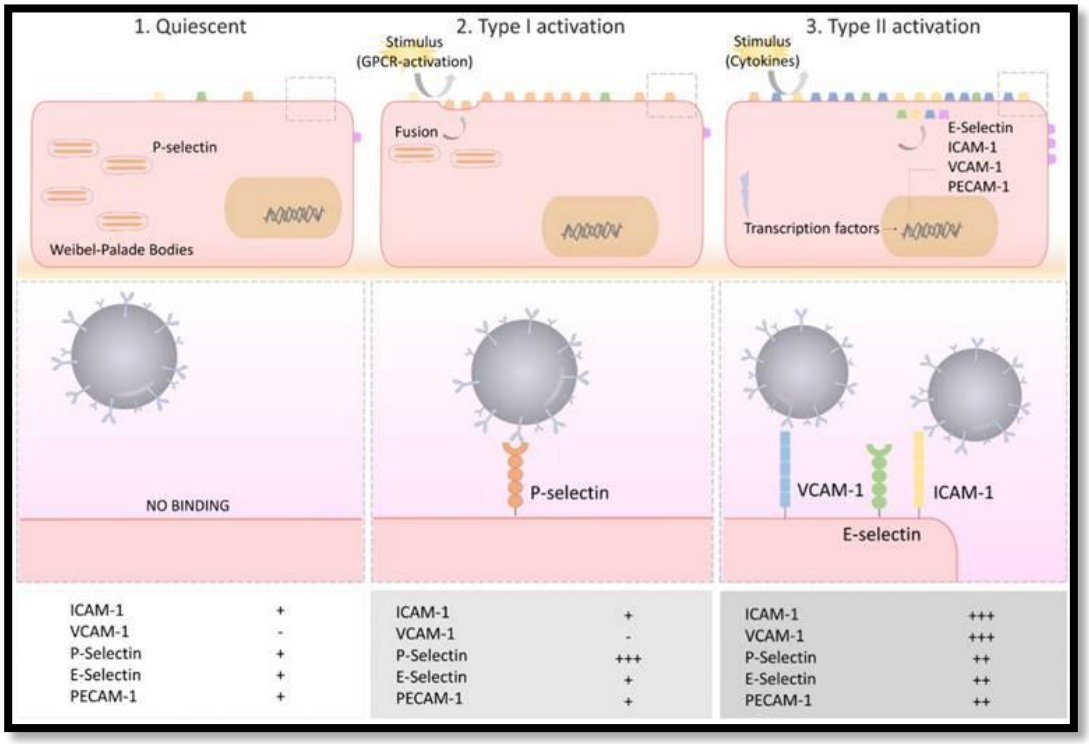


Figure 2.5. Schematic representation of (1) quiescent, (2) type I activated, and (3) type II activated endothelial cells, together with selectin and adhesion molecule expression profiles. Image used under the terms of the Creative Commons Attribution (CC BY-NC 4.0) license (https://creativecommons.org/licenses/by-nc/4.0/) (Gauberti *et al.* 2018)

In contrast, type II endothelial activation results in a more extensive and long-term phenotypic changes in endothelial cells. This type of activation can be initiated by bacterial elements such as lipopolysaccharide (LPS) and cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Hansson & Libby, 2006). Activation of the endothelium by these factors activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This pleiotropic transcription factor increases the luminal expression of ICAM-1, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), IL-8 and monocyte chemoattractant protein-1 (MCP-1). Importantly

VCAM-1 has particular specify for monocytes and lymphocytes and is one of the earliest endothelial receptors expressed in early atheromatous plaques (Hansson & Libby, 2006). Additionally, activated endothelial cells and incoming monocytes and lymphocytes secrete further proinflammatory factors such as IL-1, MCP-1, and reactive oxygen species which generates a localised positive feedback loop in the blood vessel. Once in the subendothelial space, monocytes become macrophages and accumulate LDL cholesterol and other cell debris to form bulky 'foam' cells. Very early lesions be detected histologically by the presence of scattered lipid-containing foam cell macrophages (Stary *et al.*, 1994). Macroscopically, these cellular aggregations appear as fatty streak, consisting of aggregates of foam-cells and lipid-containing smooth muscle cells, but lacking extracellular deposits. The adjacent VSMCs also undergo a phenotypic switch from a predominantly contractile function to a synthetic and proliferative function and also contribute to lumen loss by secretion of collagen and other proteoglycans (Ross, 1993). With further recruitment of chronic inflammatory cells such as monocytes, lymphocytes, dendritic cells and mast cells luminal diameter becomes constricted limiting the flow of blood beyond the lesion. As lesions progress over years, lipid accumulation and cellular proliferation further constrict luminal diameter and lesions are known as atheromatous plaques (Stary *et al.*, 1995; Tabas & Bornfeldt, 2016).

In summary, the normal homeostatic functions of endothelium are to regulate vascular tone, prevent inflammatory cell adhesion and intravascular thrombosis. In contrast, 'endothelial dysfunction' summarises the non-adaptive responses of the endothelium which promote vasoconstriction, inflammatory cell accumulation

and activation and thrombosis that are central to the initiation and progression of atherosclerosis.

#### 2.3.2.9 **Endothelial Dysfunction and its Assessment**

The dysfunctional endothelial phenotype can be identified in vivo by abnormal vasoconstriction in response to intracoronary administration of normally vasodilating acetylcholine (Ludmer *et al.*, 1986). This is recognised as the gold standard for assessment of endothelial function, however is limited due to its invasive nature, cost and resource utilisation (Flammer *et al.*, 2012). A non-invasive means of evaluating peripheral endothelial function was first introduced by Celermajer and Deanfield when they devised a method that measures the change in the diameter of the brachial artery in response to downstream reactive hyperaemia termed, flow-mediated dilatation (FMD) (Celermajer *et al.*, 1992). Flow mediated dilatation by this method is defined as the maximal percent change in conduit vessel diameter in response to the hyperaemic stimulus. In this method, a five-minute period of ischaemia caused by an arterial occlusion cuff causes vasodilation of downstream vascular beds and thus a hyperaemic response (Figure 3.1 and Figure 3.3). Upon release of the cuff blood flow into the ischaemic area via the conduit arteries is increase which imparts a relative increase in laminar shear-stress on the endothelial wall. This increased shear-stress stimulates the endothelial release of nitric oxide which results in vasodilation of the conduit artery. Blunted or absent dilatation was shown in adults with coronary artery disease or who were smokers, or children with risk factors such as familial hypercholesterolaemia, compared to controls ($p<0.001$) (Celermajer *et al.*, 1992, 1993). Subsequently, the mechanism behind FMD was shown to be due to the

release of nitric oxide from the endothelium (Joannides *et al.*, 1995; Meredith *et al.*, 1996). Practically, brachial FMD correlated strongly with the gold standard method of intracoronary acetylcholine responses (Takase *et al.*, 1998) and both have been shown to be an independent predictors of atherosclerotic disease progression and cardiovascular events (Schächinger, Britten & Zeiher, 2000). Among patients with PAD, abnormal brachial FMD was shown to be an independent predictor of long-term cardiovascular events (Brevetti *et al.*, 2003; Gokce *et al.*, 2003), but also a predictor of walking disability (Grenon *et al.*, 2014).

In the literature, various methods have been used to measure FMD, which is generally performed on the upper limb, but can also be performed on the lower limbs (Kooijman *et al.*, 2008). Upper limb versus lower limb FMD assessments are probably not equivalent, because of significant differences in vascular anatomy, but this has not been demonstrated. Most protocols are consistent in advocating a period of rest, fasting, abstinence from cigarette smoking, and temporary withholding of vasoactive medications because these factor can affect the FMD response to hyperaemia (Corretti *et al.*, 2002; Thijssen *et al.*, 2011). Assessment of FMD is technically challenging and requires knowledge and skill in the use of vascular ultrasonography. Early methods required the sonographer to hold the probe fixed in position for the duration of the test which is difficult to do. Subsequent groups recommended stereotactic probe holders, with micrometre adjustment, which eliminate the need for continuous operator contact and probably improves the accuracy and reproducibility (Donald *et al.*, 2008). Early, methods for measuring brachial artery diameter were also rather crude and utilised the on screen calliper function which are subject to significant intra-observer

variability (Celermajer *et al.*, 1994). As such, it has been suggested that a minimum of 100 supervised scans are needed to achieve independent competency, and a minimum of 100 scans per year to maintain competency (Corretti *et al.*, 2002). Edge detection software has been shown to reduce intra-observer variability (Woodman *et al.*, 2001) and is more sensitive in detecting small changes in arterial diameter however such software packages can cost upwards of $US 5,000. Nevertheless, adherence to the current expert consensus guidelines and use of adjunctive methods such as stereotactic probe holder and validated edge detection software significantly reduces measurement error (Greyling *et al.*, 2016).

Another factor needing consideration when evaluating the data on exercise and FMD in patients with claudication, is position of the occlusion cuff during the period of ischaemia which can be placed on the proximal arm above the imaged artery, or on the forearm (Corretti *et al.*, 2002). This different approach to cuff position during FMD measurement has been shown to result in a different FMD response, with the proximal cuff technique invoking a dilation response that is larger but only partially attenuated by the administration of a NO synthase inhibitor, whereas the distal occlusion response was lower but totally abolished by administration of the inhibitor (Doshi *et al.*, 2001). This suggests the distal occlusion method elicits a more valid measurement of endothelial function than the proximal cuff occlusion method, and is thus recommended method in published technical guidelines (Thijssen *et al.*, 2011).

#### 2.3.2.9.1 **Other Non-Invasive Methods for Assessing Endothelial Function**

Peripheral artery tonometry (PAT) is an alternative non-invasive technique believed to provide an assessment of endothelial function (Nohria, 2006). Peripheral artery tonometry utilises fingertip pneumoplethysmography (Itamar Medical Ltd., Caesarea, Israel)) to measure digital pulse wave amplitude at rest and following a 5-minute ischaemic stimulus induced by brachial artery occlusion (see Figure 3.4 and Figure 3.5). The PAT reactive hyperaemia index (RHI) is defined as the ratio of the average pulse wave amplitude over 1 minute, beginning after exactly 1 minute after cuff deflation, to the average pulse wave amplitude recorded during the baseline period (Kuvin *et al.*, 2003). This ratio is believed to be reflective of endothelial and NO dependent vasodilation (Noon *et al.*, 1996). It has been shown to correlate with coronary FMD, and is lower in asymptomatic patients with risk factors compared to those with coronary artery disease (Kuvin *et al.*, 2003). In addition, cross-sectional evidence suggests that RHI is predictive of late adverse cardiac events in a prospective cohort of 270 patients (Hamburg *et al.*, 2008; Rubinshtein *et al.*, 2010).

The Vendys2 device (Endothelix Inc, Houston, TX) is an analogous system to the PAT that uses fingertip temperature sensing probes to measure the digital temperature change in response to cuff-occlusive reactive hyperaemia. It has also been shown to lower in patients with coronary heart disease or who have high Framingham risk score (Gul *et al.*, 2009). Both systems are automated and are not operator dependent which in theory should increase the reliability, however PAT has actually shown to have almost twice the within day variability compared to FMD (Onkelinx *et al.*, 2012). Compared to FMD, which detects the vasomotor response in medium sized conduit arteries, PAT-RHI detects the hyperaemic

response in microvascular beds. Also the occlusion cuff is place above the cubital fossa and it is speculated that this invokes myogenic and neurogenic components to increase hyperaemia, thus it may not represent a endothelial or NO specific test. Given the quite different methods of detecting a hyperaemic response, it is not clear whether these are useful tests in the setting of macrovascular peripheral artery disease. One small cross sectional study of patients with PAD showed that ABPI correlated independently with the RHI ($\beta = 0.254$, $p=0.041$) (Igari *et al.*, 2016), however a larger study of patients with CAD, PAD and healthy controls showed that PAD was significantly associated with impaired FMD in multivariable models ($p<0.0001$), not with RHI ($p=0.43$) (Kiani *et al.*, 2013). At present, FMD remains the most widely used and seemingly robust non-invasive tool to assess endothelial function in clinical studies.

#### 2.3.2.9.2 **Circulating Markers of Endothelial Function**

As markers of endothelial function, blood markers have great potential since they are easily accessible and allow repeatable measurements for research and clinical application. Measurement of blood NO is an intuitive starting point given its central role in endothelial function. However, its application in endothelial function testing is limited by several factors including it's highly reactive nature and extremely short half-life (Rassaf, Feelisch & Kelm, 2004). Therefore, surrogate markers of NO bioavailability have consisted of the metabolic by products which tend to be stable and excreted via known pathways (Tsikas, 2009). Nitrate and nitrite are two by-products of NO metabolism which can be measured in plasma serum and urine samples (Tsikas, 2009), with approximately 70% of plasma nitrite estimated to originate from eNOS function (Kleinbongard *et al.*, 2003). Assayed levels of nitrite and nitrates are typically converted to a total NO concentration (Delaney & Spark, 2016). A small study has shown that plasma

nitrite is decreased with increasing presence of cardiovascular risk factors and is significantly associated with brachial FMD (Kleinbongard *et al.*, 2006). It has also been shown to be significantly reduced in patients with PAD compared to controls and plasma nitrite flux has been shown to predict both severity of vascular disease and exercise capacity (Allen *et al.*, 2009). However, there remain concerns with regard to its reproducibility and confounding due to wide variation in dietary NO (Wang *et al.*, 1997; Allan, Vun & Spark, 2016)

.

Asymmetric dimethylarginine (ADMA), the endogenous competitive inhibitor of L-arginine at eNOS, has been shown to be associated with the presence of cardiovascular risk factors, disease and NO bioavailability (Vallance & Leiper, 2004; Sibal *et al.*, 2010). Furthermore, it has been shown to be predictive of brachial artery flow mediated dilatation (Päivä *et al.*, 2010) and correlate independently with severity of PAD and is predictive of major adverse cardiac events (Wilson *et al.*, 2010; Staniszewska *et al.*, 2015). Although it is a relatively expensive test is can be easily and precisely measured using a traditional ELISA assay (Schulze *et al.*, 2004).

Other molecules that can be easily measured using ELISA techniques include those that are released by endothelial cells which have been activated. Such molecules include the adhesion molecules which trigger leukocyte adhesion and translocation during inflammation for example E-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and P-selectin (Ridker, 2004). Expression of these molecules is inhibited by NO, therefore they may serve as surrogate markers of endothelial function (De

Caterina *et al.*, 1995). However, many are non-specific and are altered by unrelated inflammatory conditions (Denton *et al.*, 1995).

Endocan (also known as endothelial cell specific molecule 1, ESM-1) is a small dermatan sulphate proteoglycan molecule touted as a novel marker of endothelial function and vascular inflammation. Endocan was originally isolated and described by Béchard *et al.* who showed that it its preferentially located in human vascular tissues and probably functions to modulate the recruitment of circulating lymphocytes and mononuclear cells under the influence of inflammatory cytokines (Béchard *et al.*, 2000, 2001). Endocan is highly expressed in atherosclerotic plaques but not in quiescent vascular endothelium and upregulates vascular smooth muscle proliferation and migration (Menon, Kocher & Aird, 2011). It has been shown to be an easily measured and stable molecule than can be accurately measured from patient blood samples by ELISA (Gaudet *et al.*, 2017). Increasing data is accumulating on the role of endocan in subclinical and overt vascular disorders. For example, endocan levels were significantly higher in a cross-sectional study of newly diagnosed hypertensive patients versus controls (Balta *et al.*, 2014). In another study of patients with renal failure and healthy control subjects, plasma endocan was independently associated with FMD on multivariable analysis, and was an independent predictor of all-cause mortality and cardiovascular events (Yilmaz *et al.*, 2014). More recent work has shown endocan levels to be significantly higher in patients with unstable coronary artery disease (Qiu *et al.*, 2016). Thus, endocan is emerging as a potentially useful circulating marker of endothelial function and atherosclerotic disease progression. However, to date, no study has looked at endocan levels in patients with PAD. Chapter 7 examines endocan levels in patients with intermittent claudication.

Other circulating markers of endothelial function that have been investigated include circulating endothelial cells and endothelial micro-particles, and bone marrow derived endothelial progenitor cells. These markers can be detected by flow cytometry techniques and have been shown to be increased in limb ischaemia (Makin *et al.*, 2004; Deanfield, Halcox & Rabelink, 2007).

### 2.3.3 Role of the Immune System and its Assessment in PAD

#### 2.3.3.1 Components of the Immune System

As already alluded to in the previous sections, the immune system consists of innate and adaptive arms that can each be further divided into cellular humoral components. The innate immune system serves as the, second line of defence after physical barriers, and consists of both cellular and humoral components (Porcelli, 2017). The humoral components of the innate immune system consist of circulating proteins such as CRP, natural antibodies and complement proteins (Shishido *et al.*, 2012). Within the circulation, these components bind to pathogens and damaged tissues and enhance phagocytosis by neutrophils and macrophages, which are cellular components of the innate immune system. Neutrophils and macrophages can also recognise antigenic molecular patterns though a large family of pattern recognition receptors called Toll-like receptors (TLRs) (Akira, Uematsu & Takeuchi, 2006). Where the antigenic pattern originates from exogenous pathogenic or infectious organisms it is termed a pathogen-associated molecular patterns (PAMPs). The prototypical example of process is during septic shock where lipopolysaccharide (LPS) molecules from gram-negative bacteria are recognised by TLR4 (along with co-receptor CD14)

leading to an intracellular signalling pathway NF-κB and inflammatory cytokine production (Lu, Yeh & Ohashi, 2008). In contrast, a tissue damage may cause the release endogenous molecules from stressed or necrotic cells which possess antigenic patters known as damage-associated molecular patterns (DAMPs). Like PAMPs, DAMPs are recognised by similar pattern-recognition receptors on neutrophils, monocytes and macrophages leading to induction of proinflammatory response via the release of cytokines. For example, TLR4 can also recognise endogenous molecules such as matrix components and heat shock proteins to trigger activation of proinflammatory pathways (Asea *et al.*, 2002; Schaefer *et al.*, 2005). Additionally, mast cells, eosinophils, and natural killer cells are also components of the cellular arm of the innate immune system, which play a role in promoting tissue inflammation and cell mediated cytotoxicity of infected, neoplastic and foreign cells (Spiering, 2015). The innate arm of the immune system in relatively rapid to act, is non-specific, and does not change with repeated exposure. In contrast, the adaptive arm of the immune system is slower to mount a response, but adapts after initial exposure to a specific antigen. Secondary adaptive immune responses are specific for an antigen and demonstrate increased magnitude of response. The primary components of the adaptive immune system are B-lymphocytes and T-lymphocytes which exists as large populations of clones that each recognise a specific antigen. The primary function of B-lymphocytes is to differentiate into antibody producing plasma cells. T-lymphocytes can be either 'helper' T-lymphocytes (differentiated by expression of CD4), which secrete cytokines to enhance the B-lymphocyte activation, or 'cytotoxic' T-lymphocytes (differentiated by expression of CD8), which destroy virus infected and neoplastic cells (Yatim & Lakkis, 2015).

The cellular components of the immune system are summarised in Figure 2.6

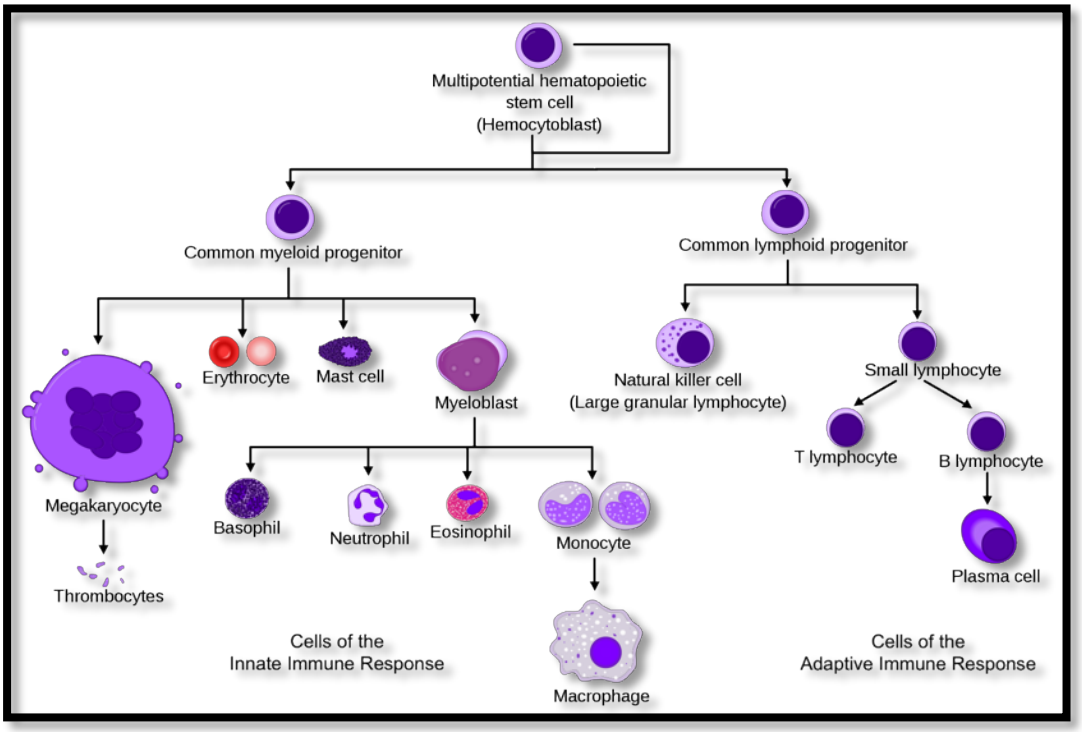


Figure 2.6. Cells of the innate and adaptive immune system.

Image used under the terms of the Creative Commons Attribution (CC BY-NC 3.0) license (https://creativecommons.org/licenses/by-nc/3.0/). (Mikael Häggström and A. Rad., 2009)

### 2.3.3.2 **The Role of the Immune System in Atherosclerosis**

Over the last 20-30 years it has been increasing recognised that atherosclerosis is an inflammatory condition of arteries initiated by the response to the retention of lipoproteins (Skalen *et al.*, 2002). Retained lipoproteins, in particular LDL, are phagocytosed by resident macrophages and undergo modification into oxidised LDL, promote the release of inflammatory cytokines (Hansson & Hermansson, 2011). Minimally modified LDL can activate the release of proinflammatory cytokines by binding to TLR4 on macrophages (Miller *et al.*, 2003). Locally produced cytokines and oxLDL itself can then activate endothelial cells resulting in the expression of luminal signalling molecules such as, P-selectin, E-selectin,

VCAM-1, ICAM-1, very-late antigen-4 (VLA-4) and PECAM-1 (Huo, Hafezi-Moghadam & Ley, 2000; Galkina & Ley, 2007). Interaction between bone-marrow derived monocytes and these endothelial surface molecules leads to the adherence, rolling, capture and eventual trafficking of these cells into the subendothelial space. Here monocytes have been shown to differentiate into M1 macrophages under the stimulation by macrophage colony-stimulating factor (M-CSF), which further enhance the proinflammatory milieu by secreting a range of proinflammatory mediators such as cytokines TNF-α, IL-6, IL-1β, and ROS (Tabas & Bornfeldt, 2016). Macrophages phagocytose extracellular lipoproteins forming foam cells which contributes to lesion expansion. Antigen presenting cells such local dendritic cells and macrophages present local antigens to T-lymphocytes that are also recruited and trafficked into the subendothelial space, leading to a $T_H1$ lymphocyte response characterised by production of TNF-α and INF-γ further amplifying the local inflammation in a form of positive feedback. Regulatory T-lymphocytes macrophages displaying a M2 response may attenuate the local inflammatory milieu by secretion of anti-inflammatory cytokines IL-10 and TGF-β, but the exact role of these cells in atherogenesis is unclear.

The role of the immune system in the progression of cardiovascular disease is supported by epidemiological studies have demonstrated strong associations between cardiovascular diseases and elevated leucocytes, in particular neutrophils (Eryd *et al.*, 2012). In PAD populations, elevated levels of CRP, D-dimer, IL-6, and soluble VCAM-1 are associated with adverse calf muscle characteristics and reduced calf muscle strength (McDermott *et al.*, 2007a) suggesting that the inflammatory milieu are associated with the functional impairment of patients with intermittent claudication. In addition, both humoral and cellular markers,

such as CRP, IL-6, and neutrophil-lymphocyte ratio, have been shown to associated with limb related disability (McDermott *et al.*, 2008a) and mortality in patients with PAD (Spark *et al.*, 2010; Chan *et al.*, 2014).

#### 2.3.3.3 **Monocyte Subpopulations**

Monocytes have long been recognised as central players in the development of atherosclerotic lesions. In 1958, Poole and Florey reported the adherence of macrophages to the aortic endothelium of rabbits fed a high cholesterol diet (Poole & Florey, 1958). Later in 1980, Gerrity *et al*. reported the morphological characteristics of early atheromatous lesions in a swine model of atherosclerosis (Gerrity, 1981). In these pigs, fatty streaks occurred at areas of increases vascular permeability. Importantly, these lesions demonstrated strong preferential adherence of monocytes to the intima of lesions was able to be identified by transmission electron microscopy. Similar findings were reported by Faggioto *et al*. in 1984, noting the loss of endothelial continuity and the adherence of monocytes to the endothelium at sites of developing atherosclerosis (Faggiotto, Ross & Harker, 1984). As mentioned above, once recruited to early lesions, monocytes undergo transformation into inflammatory macrophages scavenging oxLDL and secreting proinflammatory cytokines.

Despite this prominent role on the monocyte-macrophage system in atherogenesis, epidemiological evidence linking circulating monocytes counts to clinical apparent atherosclerotic disease has been conflicting. For example, a French cohort study of adult males prospectively followed between 1980 and 1989 found that a high monocyte count was strongly associated with the risk of coronary artery disease independent of smoking and other classical risk factors (Olivares,

Ducimetière & Claude, 1993).Two other studies have found monocyte levels predictive of cardiovascular events, (Horne *et al.*, 2005; Dragu *et al.*, 2008), whilst another found no association (Rana *et al.*, 2007). However, a much larger study consisting of a meta-analysis of seven prospective cohort studies, incorporating 1,764 incident cases of coronary artery disease among 30,374 patients, found no significant association with monocyte counts (Wheeler *et al.*, 2004). It has been hypothesised that the monocyte heterogeneity might explain the lack of a relationship between monocyte levels and cardiovascular complications (Hilgendorf & Swirski, 2012).

In murine models of atherosclerosis, monocytes are able to be distinguished into two subsets by the surface expression of glycoprotein Ly6C (Geissmann, Jung & Littman, 2003). Murine monocytes with high Ly6C expression ($Ly6C^{high}$) have been observed to increase rapidly in the blood and adhere to activated endothelium in a model of atherosclerosis (Swirski *et al.*, 2007). Furthermore, $Ly6C^{high}$ monocytes infiltrate atherosclerotic lesions and become resident macrophages. Unsurprisingly, $Ly6C^{high}$ monocytes are characterised by a proinflammatory phenotype and produce high amounts of TNF-α (Crane *et al.*, 2014). In humans, monocytes can similarly be distinguished based on the surface expression marker molecules.

Before 1989, human monocytes were considered to be a relatively homogenous population of circulating cell. During 1989, Passlick *et al*. described two subpopulations of human monocytes based on the surface expression of CD14 and CD16 (Passlick, Flieger & Ziegler-Heitbrock, 1989). CD14 is a transmembrane cell surface receptor found on many cells of the innate immune system that acts as

a co-receptor (along with TLR4) for the detection of bacterial lipopolysaccharide (LPS) and other PAMPs (Wright *et al.*, 1990). Binding of CD14 and TLR4 results in the activation of proinflammatory transcription factors such as NF-κB (Muller, Ziegler-Heitbrock & Baeuerle, 1993). On the other hand, CD16 is a receptor for Fc-gamma portion of IgG antibodies that mediates antibody dependent cell-mediated cytotoxicity (Yeap *et al.*, 2016). Initially, human monocytes were divided into classical monocytes if they were $CD14^{++}/CD16^{-}$, and $CD16^{+}$ monocytes. CD16-positive monocytes were further characterised by relatively high expression of MHC II, which is important in initiating immune responses through the presentation of antigens to other cells of the adaptive immune system (Roche & Furuta, 2015). In addition, CD16-positive monocytes demonstrated relatively higher expression of TNF-α expression following ligation of TLR receptors in vivo (Belge *et al.*, 2002). A more recent definition was proposed in which monocytes can be characterised into three subsets based on CD14 and CD16 expression that has been recognised by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock *et al.*, 2010). There are: (1) classical monocytes $CD14^{++}/CD16^{-}$, (2) intermediate monocytes $CD14^{++}/CD16^{+}$, and (3) non-classical monocytes $CD14^{+}/CD16^{++}$. Classical monocytes account for 85-90% of the circulating monocyte population, whereas intermediate monocytes count for approximately 5%, and non-classical approximately 10% (Boyette *et al.*, 2017) .

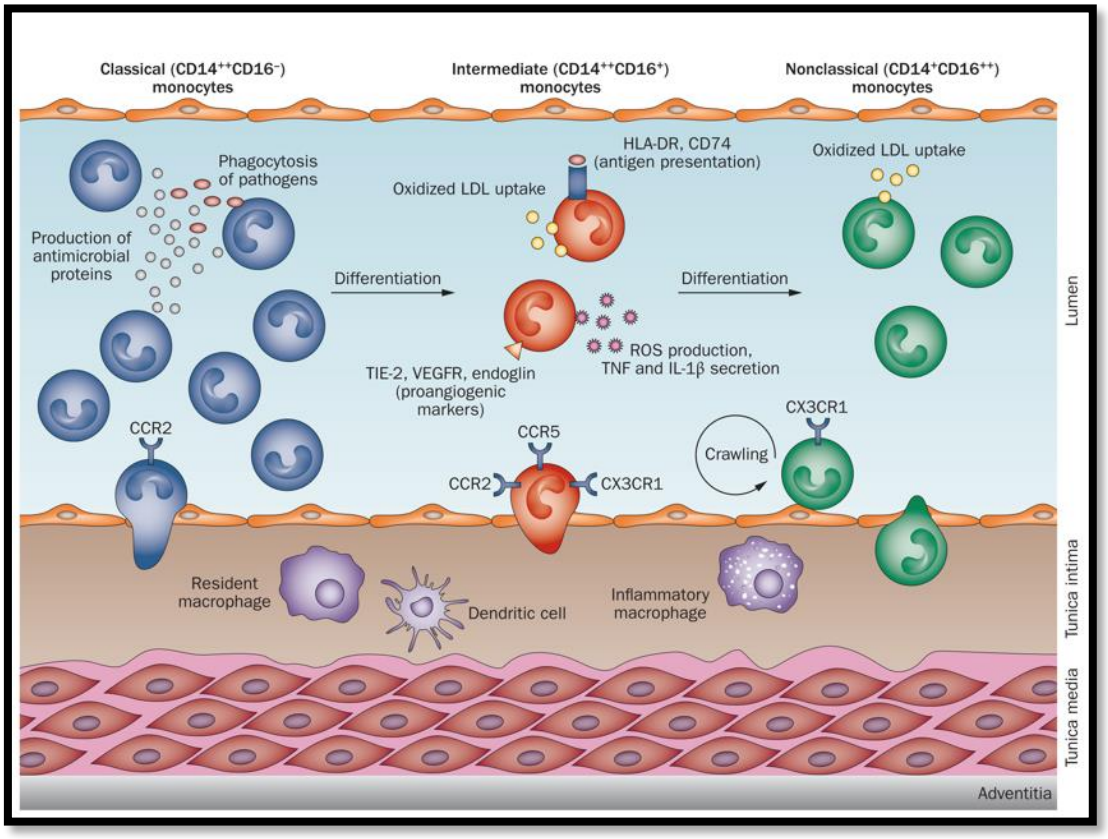


Figure 2.7. The potential functional roles of monocyte subsets, as suggested by gene expression and functional studies. Image used with permission from Springer Nature (Heine *et al.*, 2012).

Research on the functional characteristics of this subset have revealed a relatively high phagocytic potential production of antimicrobial agents (Zawada *et al.*, 2011), strong interactions with platelets (Urra *et al.*, 2009), and production of monocyte chemoattractant protein-1 (MCP-1) and the anti-inflammatory cytokine IL-10 (Cros *et al.*, 2010). In contrast, intermediate monocytes, which may represent an activated form of classical monocytes, display a relatively high degree oxLDL uptake (Mosig *et al.*, 2009), high MHC II expression for antigen presentation (Rossol *et al.*, 2012), high basal production of ROS (Zawada *et al.*, 2011) and a proinflammatory response with secretion of TNF-α (Schlitt *et al.*,

2004), IL-6, and IL-1β. (Belge *et al.*, 2002; Ancuta, Wang & Gabuzda, 2006). Intermediate monocytes may further differentiate into non-classical monocytes which demonstrate patrolling behaviour at the blood-endothelial interface via the interactions between monocyte chemokine receptor CX3CR1 and the endothelial ligand CX3CL1 (fractalkine), which may result in the subendothelial retention of monocytes (Ancuta *et al.*, 2003). The potential functional roles of monocyte subsets are summarised in Figure 2.7.

#### 2.3.3.3.1 **Clinical Significance of Monocyte Subpopulations**

In light of these differential functions, it could be hypothesised that monocyte subsets each play a different role in pathogenic processes. Classical monocytes appear to display characteristics well suited to respond to bacterial pathogens and infections. Conversely, intermediate and non-classical monocytes display functional characteristics that are associated with the pathogenesis of atherosclerosis, namely, endothelial adhesion, ROS production, and inflammatory cytokine production. These functional characteristics are likely to be critical in the development of atherosclerosis.

Whilst there is no direct clinical evidence of the roles of these monocyte subtypes in atherosclerotic disease, epidemiologic studies have supported these hypotheses, highlighting their likely relevance to cardiovascular disease. For example, in a case-control study of patients with coronary artery plaques, those with vulnerable plaques had $CD14^{+}/CD16^{+}$ monocyte proportions were significantly greater relative to patients without vulnerable plaques and healthy control subjects (Kashiwagi *et al.*, 2010). Similarly, a prospective study of non-dialysis CKD patients followed for 5 years found that increasing intermediate $CD14^{++}/CD16^{+}$ monocytes independently predicted the occurrence of adverse cardiovascular

events (per 10cells/μL increase, HR 1.26 (1.04-1.52, p=0.018)) (Rogacev *et al.*, 2011). Again, in a study of 951 patients presenting for elective coronary angiography, intermediate monocyte levels were demonstrated to be independently associated with cardiovascular events even after full multivariable adjustment (Rogacev *et al.*, 2012). In PAD cohorts, patients with intermittent claudication or critical limb ischaemia, higher proportions of intermediate and non-classical monocytes than normal controls (Dopheide *et al.*, 2012), suggesting that they might play a role in the pathogenesis. Importantly for patients with claudication, high levels of intermediate monocytes may contribute to walking performance since it was recently shown that high levels of monocytic expression of TNF-α mRNA the correlate negatively with maximal walking time (Pande *et al.*, 2015). A more recent cross-sectional study of 143 patients with various stages of PAD observed a significant positive correlation between intermediate monocytes $CD14^{++}CD16^{+}$ and increasing Rutherford stage of PAD (Wildgruber *et al.*, 2016a). Whilst these studies can't establish mechanistic causality, they do provide circumstantial evidence that implicates intermediate monocytes in the progression of cardiovascular disease.

#### 2.3.3.3.2 **The Effect of Exercise on Monocyte Subpopulations**

The effect of exercise on monocyte subtypes has been investigated since the first realisation of their existence. Steppich *et al.* noted that strenuous exercise in healthy adults led to a doubling of the absolute numbers of $CD14^{+}CD16^{+}$. Monocytes determined by two-colour flow cytometry (Steppich *et al.*, 2000). Hong and Mills subjected 44 volunteers, to 20 minutes of treadmill exercise finding that all monocyte subtypes increased in absolute terms, but the proportions of $CD14^{++}/CD16^{-}$ decreased, whereas $CD14^{++}CD16^{+}$ and $CD14^{+}/CD16^{++}$

subtypes increase significantly (Hong & Mills, 2008). Later, Heimbeck *et al.* found similar results in a cohort of healthy adults after strenuous exercise led to a greater than three-fold increase in the absolute numbers of $CD14^{+}/CD16^{++}$ monocytes determined with four-colour flow cytometry (Heimbeck *et al.*, 2010). More recently, LaVoy *et al*. reported that in healthy volunteers, maximal treadmill running was associated with a near 3-fold increase in absolute total monocytes, together with a more than doubling of the proportions of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes (LaVoy *et al.*, 2015). These consistent results demonstrate that, at least in healthy individuals, acute exercise appears increase the proportions of what would be defined as CD16-positive intermediate and nonclassical monocytes.

Chronic training, however, appears to lower the number of $CD16^{+}$ monocytes. Timmerman *et al.* exercised sedentary adults for 3 days per week for 12 weeks and measured $CD16^{+}$ monocyte levels and compared this to a similar group of physically active adults who maintained their baseline physical activity (Timmerman *et al.*, 2008). Interestingly, at baseline, sedentary participants had a higher proportion of $CD16^{+}$ monocytes compared to the physical active group. In addition, these cells produced greater amounts of TNF-α. The period of exercise was, however, able to decrease the proportion of $CD16^{+}$ monocytes in the sedentary group. Furthermore, this was associated with a reduction in stimulated monocyte TNF-α production. These results demonstrate an anti-inflammatory modulation of monocytes through chronic exercise.

Together this highlights monocyte driven inflammation as a potential pathologic process and therapeutic target in intermittent claudication. The effects of exercise therapy on immune function and inflammation are reviewed in section 2.4.3.4.4.

#### 2.3.3.4 **microRNAs**

It is well known that the genes coding for cellular proteins and enzymes are contained in the in a cell's DNA (Crick, 1970). Genes in turn, code for mRNA, which are in turn translated by ribosomes into a functional or structural protein. Individual genes, however, make up only about 1.5% of the entire genome (He & Hannon, 2004).

The remaining 98% of the genome is increasingly understood to possess non-gene regulatory nucleotide sequences, which can modulate the translation of mRNAs to proteins. These regulatory non-gene RNAs, which are now known as microRNAs, were first discovered whilst studying the larval development of the nematode *Caenorhabditis elegans* (Lee, Feinbaum & Ambros, 1993). Lee *et al.* found that a small hair-pin loop of RNA (lin-4), 22-61 nucleotides long, could negatively regulate lin-14 mRNA, and thus inhibit lin-14 protein expression through a complimentary RNA-RNA interaction. Since then many more miRNAs have been discovered and characterised in organisms such as animals, plants, and viruses. To catalogue newly discovered miRNAs, a bioinformatics repository of miRNAs, miRbase, was created by Sam Griffith-Jones in 2002 and at the time contained just over 200 entries (Griffiths-Jones, 2004). The database has grown rapidly (Figure 2.8) and the current version 22, released in March 2018, has 38,589 miRNA entries, of which, 2,654 entries are for mature miRNAs found in *Homo sapiens* (miRBase, 2018).

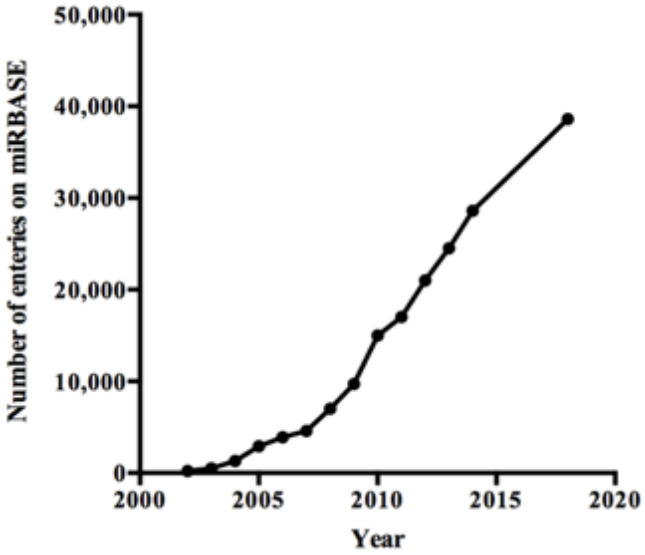

Figure 2.8. Individual microRNA entries on miRbase from inception to 2018 (Griffiths-Jones, 2018).

#### 2.3.3.4.1 **microRNA physiology**

An eloquent review of miRNA biogenesis and physiology has been published by Kim and what follows is a brief summary of this (Kim, 2005).

Within the nucleus of cells, microRNAs are transcribed from the DNA by DNA polymerase II, which is the same polymerase that creates mRNA, creating a primary RNA transcript with a hair-pin loop. Two endonucleases, Drosha and Pasha, are able to recognise part of the primary RNA transcript and cleave off part a portion of the molecule leaving a "pre-miRNA" hair-pin molecule. The pre-miRNA molecule subsequently exits the nucleus via, exportin-5 and finds itself in the cytoplasm where it is further modified by the ribonuclease, Dicer, which removes the hair-pin loop, leaving a pre-miRNA duplex of complimentary RNA stands. An intracellular protein knowns argonaute has a binding domain that recognises to the 3' end of miRNA regardless of the sequence. Once argonaute combines with one of the miRNA strands, it forms a RNA-induced silencing complex (RISC), which can either mark a mRNA for cleavage, destabilise it by shortening its poly-adenylated tail, or repress translation by ribosomes (Bartel, 2004, 2009) (Figure 2.9). The physiological impact of this function at the cellular level is on gene expression, given that degraded mRNA sequences are no longer available for translation into proteins by ribosomes.

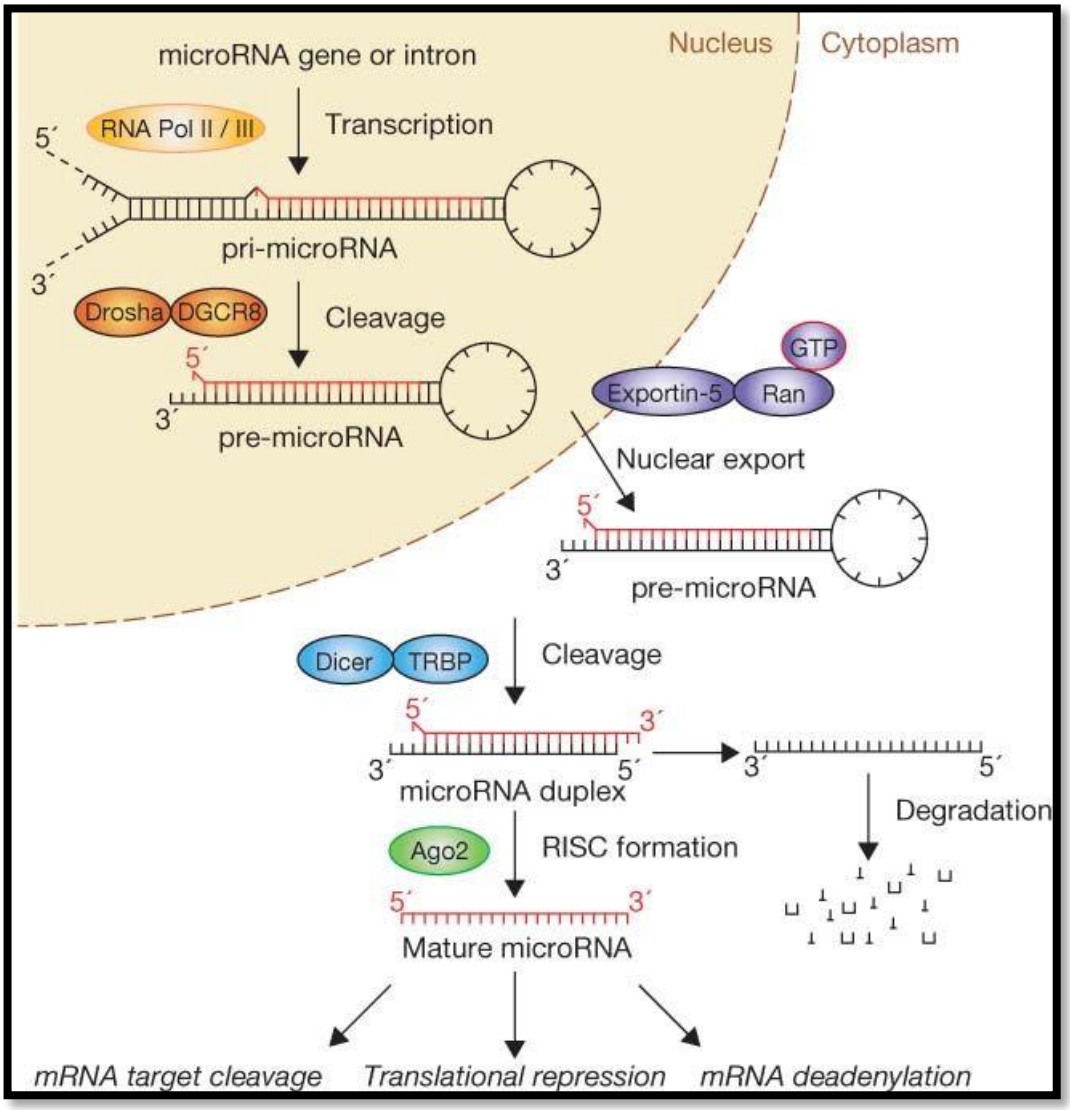


Figure 2.9. The miRNA processing pathway. Image used with permission from Springer Nature: Nature Cell Biology (Winter *et al.*, 2009).

#### 2.3.3.4.2 **Role of microRNAs in Cardiovascular Disease**

A number of microRNAs have been implicated in a diverse range of human disease, first in leukaemia (Calin *et al.*, 2002), and later in cardiac disease (van Rooij *et al.*, 2006; van Rooij, 2011). In the field of PAD, one early clinical case-control study from China identified miR-130a, miR-27b and miR-210 as serum biomarkers in patients with atherosclerosis obliterans (Li *et al.*, 2011). Not only were the level of these miRNAs increased in patients versus controls, but they

also were significantly higher in those with greater disease severity. The role of these miRNAs in PAD is not fully understood, but is possibly linked to neo-angiogenesis (Chen & Gorski, 2007; Fasanaro *et al.*, 2008).

In a number of animal models, specific miRNAs have been implicated in regulation of endothelial function, vascular remodelling and neo-angiogenesis. For example, Bonauer *et al*. demonstrated that miR-92a was highly expressed in human umbilical vein endothelial cells (HUVECs) and appeared to inhibit the angiogenesis when overexpressed in an in vitro study. Conversely, inhibition of miR-92a with a pharmaceutical "antago-miR" improved angiogenesis and tissue recovery in a mouse model of hind-limb ischaemia in vivo (Bonauer *et al.*, 2009). In a subsequent large animal model of ischaemia-reperfusion injury in pigs, regional delivery of a miR-92a inhibitor, reduced infarct size, inflammatory response and increased capillarisation (Hinkel *et al.*, 2013a). Additionally, steady laminar blood flow, widely considered to be protective against atherosclerosis, has been demonstrated to downregulate miR-92a expression, whilst turbulent flow leads to upregulated expression (Wu *et al.*, 2011). Wu *et al*. also demonstrated that inhibition of miR-92a levels are critical to maintaining the expression of eNOS and thrombomodulin which are both critical in maintaining endothelial function (see Figure 2.10). Conversely, Loyer *et al*. have shown that, in mice, the combination of low haemodynamic shear-stress and oxLDL promotes the endothelial activation and atheroma formation, and this can be reduced by inhibition of miR-92a (Loyer *et al.*, 2014). miR-92a has been shown to target the transcription factors Krüppel-like Factor (KLF) 2 and 4 which normally function to upregulate eNOS and thrombomodulin expression and repress the proinflammatory NF-κB transcription factor (Lin *et al.*, 2005). In addition, KLF2

also inhibits the expressions promoters of vascular inflammation VCAM-1, E-selectin, which contributes to endothelial dysfunction (SenBanerjee *et al.*, 2004).

miRNA-92a has also be implicated in the response to physical artery injury. Daniel *et al.* demonstrated, in a femoral artery injury model in mice, systemic inhibition of miR-92a accelerated endothelial healing after denudation injury (Daniel *et al.*, 2014). A similar result occurred when miR-92a was genetically deleted from endothelial cells in mice. In both experiments, the enhanced endothelial recovery was associated with an attenuated inflammatory infiltration and neointimal formation. Thus, from the above studies it is possible that miRNA-92a is an important regulator in human peripheral artery disease and may be an effective therapeutic target. Unfortunately, there is very little published data regarding specific microRNAs in humans with PAD and no previous work on miRNA-92a in humans with PAD.

Given miR-92a expression appears inversely related to shear-stress, exercise by virtue of increase blood flow and shear-stress is likely to also impact miR-92a expression and the factors regulated by it as described above. Thus, investigating the role of miR-92a in exercising patients with claudication may provide greater insights to the function of miR-92a in vascular pathophysiology.

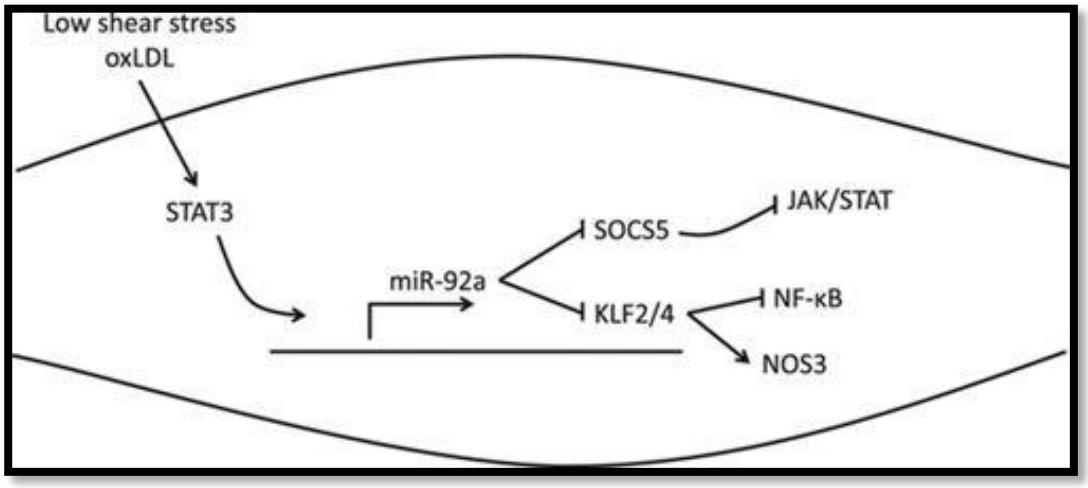


Figure 2.10. miR-92a negatively regulates KLF2/4 and thus eNOS (NOX3) expression in endothelial cells. miR-92a itself is upregulated by oxLDL and low shear-stress, thus may be negatively regulated by increased exposure to high shear-stress in exercise. Image used with permission from Wolters Kluwer Health, Inc. (de Winther & Lutgens, 2014).

Other miRNAs that may be important endothelial function are miR-21. An in vitro study that exposed HUVECs to unidirectional shear-stress found 13 miRNAs that were upregulated (Weber *et al.*, 2010). Of these, miR-21 demonstrated the largest magnitude change (5.2 fold, p=0.002) compared to control cells. Additionally, experimentally induced miR-21 overexpression was able to increase eNOS phosphorylation and overall NO bioavailability. More recently, miR-21 deficiency in macrophages has been implicated in vascular inflammation, atherogenesis and adverse plaque remodelling in mice (Canfrán-Duque *et al.*, 2017). Thus miR-21 is likely an important miRNA, which may be able to the modulated by exercise. Another more recent murine study has demonstrated that miR-16 is released after experimental hind-limb ischaemia, which exacerbates remote angioplasty balloon endothelial injury (Sorrentino *et al.*, 2018). Nitric

oxide bioavailability and VEGF are negatively regulated by miR-16, so interventions which antagonise it may improve endothelial function and overall vascular health. In rats, aerobic exercise training appears to downregulate the expression of cardiac miR-16, thus it could be speculated that exercise in humans exercise might have similar beneficial effects.

### 2.3.4 **Role of Skeletal Musculature and its Assessment**

Muscle dysfunction clearly plays a role in the pathophysiology of intermittent claudication with numerous studies documenting structural and function changes in the muscles and nerves of patients with claudication. The next section reviews normal muscle structure and function and alterations due to PAD and exercise therapy.

#### 2.3.4.1 **Normal Structure and Function of Skeletal Muscle**

In the adult, the skeletal muscles receive about 16% of the total cardiac output and make up approximately half of the total boy mass (Barrett & Ganong, 2012). All voluntary movement is dependent on skeletal muscle which is innervated by the spinal motor neurons of somatic arm of the nervous system. Individual skeletal muscles, of which there are approximately 600 in the human body, are collections of long multinucleated cells knowns as muscle fibres. Each muscle fibre is made up of bundles of smaller units called fibrils enclosed in a cell membrane known as sarcolemma. In addition to fibrils, the cytoplasm of the muscle fibre contains abundant mitochondria which function to aerobically generate usable energy in the form of ATP, and a sarcoplasmic reticulum (SR) which store calcium ions needed for the contractile mechanism. Fibrils are collections of myofilaments of actin and myosin, which are the basic structural units of the contractile machinery. Actin and myosin filaments have a particular arrangement such that actin and myosin filaments interdigitate in a structure known as sarcomere (van Leeuwenhoek, 1710).

Contraction of skeletal muscle is believed to occur due to the energy-dependent sliding of actin and myosin past each other in a model known as the “Sliding

Filament Theory" (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954). This model of muscle contraction is dependent on the interactions between the actin and myosin-II filaments, and two associated proteins: tropomyosin and troponin-I and energy in the form of ATP. When an action potential reaches the motor endplate of a motor nerve, the neurotransmitter acetylcholine is released causing the depolarisation of the muscle fibre. Depolarisation of the skeletal muscle fibre causes release of calcium ions stored in the sarcoplasmic reticulum within the muscle fibre. Calcium ions bind the protein troponin-I which results in a conformational change of tropomyosin and the uncovering of myosin-II binding sites on actin filaments (Lehman, Craig & Vibert, 1994). Myosin-II filaments bound to an ADP and a phosphate molecule bind to the exposed sites on actin causing the release of the phosphate. Potential energy stored in the myosin-II filament is converted into kinetic energy that causes the actin filaments to be pulled towards the centre of the sarcomere resulting in sarcomere shortening. This shortening is known as the power stroke. The ADP molecule is released during the power stroke being replaced with a new ATP molecule which is used to restore the potential energy in the myosin-II filament. In the presence of ongoing elevated levels of calcium ions and sufficient ATP, myosin-II binding sites on actin remain exposed and the contraction cycle repeats. As the muscle cell membrane potential tends to repolarise in the absence of further signals from the motor nerve, voltage-gated ion channels of the SR begin to close slowing the release of calcium ions into the cytoplasm. Additionally, ATP-dependent calcium ion pumps sequester the calcium ions from the cytosol into the SR, thus lowering the concentration of calcium ions. This reverses the conformational change in tropomyosin causing the myosin binding sites the actin myofilaments to be re-shielded preventing cross bridge formation and causing muscle relaxation.

#### 2.3.4.2 **Normal Muscle Metabolism**

As mentioned in the previous section, a continuous supply of ATP is required for muscle contraction. In muscle cells only a limited supply of ATP is readily available for muscle contraction the regeneration of ATP molecules is vitally important and there are three important ways in which this occurs. Firstly, during periods of low activity when ATP is created in excess in the resting state, ATP combines with the small molecule creatine to form phosphocreatine and ADP. The potential energy of the ATP molecule is thus stored in the phosphocreatine. When ATP levels falls rapid at the start of muscle contraction, the stored energy in phosphocreatine is rapidly converted back to ATP through the action of creatine kinase. In this way, the phosphocreatine behaves similar to a buffer to prevent large and rapid changes in ATP levels. This process, however, can provide ATP for a only a few seconds. The second mechanism to replenish ATP is through the non-oxygen dependent process of glycolysis. In this relatively rapid process, which occurs outside the mitochondria, one molecule of glucose can be converted to two molecules of ATP and two molecules of pyruvate. In the absence of oxygen, the pyruvate molecules are converted to lactic acid which when accumulates, contributes to muscle fatigue and pain. If oxygen is provided, however, pyruvate produced during anaerobic glycolysis can enter the mitochondria and undergo metabolization via the citric acid cycle. Finally, the most efficient process of ATP production is via aerobic respiration, which is the consists of the citric acid cycle and oxidative phosphorylation. During aerobic respiration one molecule of glucose is metabolized in the presence of oxygen into 36 molecules of ATP, $CO_2$, $H_2O$ and heat. The vast majority of ATP used during resting or moderately active skeletal muscle is derived from aerobic respiration

taking place in the mitochondria highlighting the important of adequate oxygen delivery through muscle perfusion.

#### 2.3.4.3 **Metabolic Changes in PAD**

In has been observed that patients in PAD suffer metabolic abnormalities in addition to haemodynamic ones. At the cellular level, a number of studies have shown that patients with claudication are prone to accumulate metabolic intermediates, such as acylcarnitines, which is thought to be a consequence of incomplete oxidization energy sources (Brass, 1996). Metabolism of fatty acids, glucose, and glucogenic amino acids yields acetyl-CoA, which can enter the citric cycle for complete oxidation. Acylcarnitines are normally created when there is an excess of acetyl-CoA that is unable to be metabolised in the citric acid cycle prior to oxidative phosphorylation in mitochondria. Excessive acetyl-CoA inhibits the enzyme pyruvate dehydrogenase, which normally catalyses the conversion of pyruvate to acetyl-CoA (see Figure 2.11). Instead, pyruvate is converted into lactate and hydrogen ions, which accumulate leading to inhibition of the actin and myosin interactions and thus muscle fatigue. Endogenous carnitine thus combines with acetyl-CoA through the action of the enzyme carnitine acetyl transferase (CAT) and buffers the high levels of acetyl-CoA, releasing CoA to be available for a number of anabolic and catabolic reactions. In patients with claudication, short-chain acylcarnitine has been observed to accumulate in the plasma during maximal exercise patients with claudication and, furthermore, elevated levels at rest are associated with poorer walking capacity (Hiatt, Nawaz & Brass, 1987). Similarly, in muscle biopsy studies of patients with intermittent claudication, high levels of short-chain acylcarnitine were negatively associated with peak exercise performance measured by $VO_{2\text{-max}}$ and treadmill-walking time. Furthermore levels

were acutely raised after a maximal walking effort in patients with claudication (Hiatt *et al.*, 1992). Together, these findings confirm the presence of metabolic muscular dysfunction in the pathophysiology of intermittent claudication.

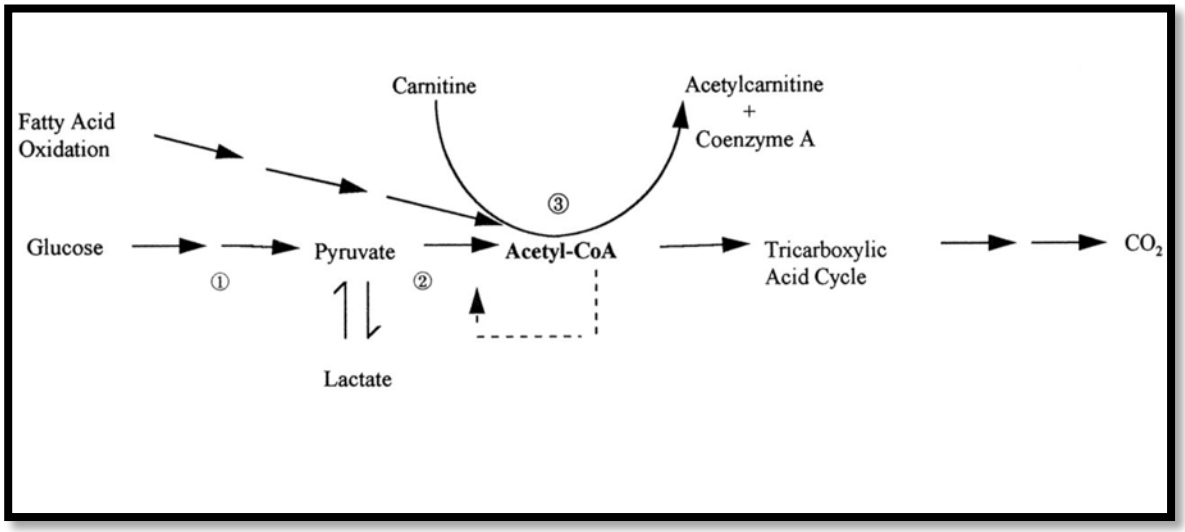


Figure 2.11. Acetyl-CoA will accumulate and can inhibit pyruvate dehydrogenase (2) in figure) leading to accumulation of lactate and hydrogen ions, the latter leading to muscle fatigue. The removal of the acetyl group from acetyl-CoA via carnitine acetyltransferase (3) can remove this inhibition freeing CoA for other methods of oxidation. Image used with permission from Taylor & Francis Group (Brass & Hiatt, 1998).

In addition to disturbed acyl-CoA metabolism, functional and structural changes in the mitochondria of skeletal muscle is also observed. In patients with intermittent claudication, the volume density of mitochondria is significantly lower when measured by transmission electron microscopy (Baum *et al.*, 2016). Clinical studies of mitochondrial enzymes can shed light on what can be described as a mitochondriopathy of PAD. Firstly, non-invasive studies using $^{31}$Phosporus magnetic resonance spectroscopy ($^{31}$PMRS) have the ability to directly measure

the ratios of phosphocreatine, ATP, and ADP before, during and after exercise or ischaemia. With this methodology, investigators have been able to calculate the recovery times phosphocreatine and ADP in skeletal muscle. An index of mitochondrial function can thus be inferred since recovery of phosphocreatine and ADP is exclusively dependent on mitochondrial function. As one could predict, patients with claudication demonstrate significantly prolonged recovery of phosphocreatine and ADP, suggesting the presence of a mitochondriopathy(Kemp *et al.*, 1995).

Corroborating evidence of mitochondrial dysfunction has been demonstrated in ex vivo clinical studies. For example, Lundgren *et al.* performed gastrocnemius muscle biopsies of patients with claudication and healthy controls and measured the level of cytochrome C oxidase (also known as Complex IV of the electron transport chain), that is a large integral protein of the inner membrane of the mitochondria critical for oxidative phosphorylative capacity (Larsen *et al.*, 2012). This study found that patients with intermittent claudication had significantly elevated levels of this mitochondrial protein, that tended to correlate with maximum walking distance. In addition, patients who underwent 6-12 months of supervised exercise augmented their levels of cytochrome C oxidase, which was suggested to account for 31% of the variability in the improvement in PFWD (Lundgren *et al.*, 1989b). However, Lundgren *et al*. also measured levels of the mitochondrial enzyme, citrate synthase (the first enzyme in the citric acid cycle), which is considered to be strongly associated with mitochondrial content (Larsen *et al.*, 2012), finding it too was elevated in patients with claudication relative to healthy controls. Similar findings were observed in another study that compared citrate synthase levels between legs of patients with unilateral claudication

(Jansson *et al.*, 1988). In contrast, however, supervised exercise training did not appear to augment citrate synthase levels in muscle. This finding was also replicated in by Hiatt *et al*. who failed to observe any change in citrate synthase levels in patients with claudication who undertook 12-weeks of supervised treadmill-walking (Hiatt *et al.*, 1996). This finding deviates from the expected increase in mitochondrial density that is normally seen in healthy individuals following endurance training (Holloszy & Coyle, 1984). Together it suggests that the mitochondrial adaptations to training are modified by the presence of peripheral ischaemia. This is unsurprising since a sufficient supply of oxygen is mandatory for the function of the mitochondrial respiration.

Ischaemic mitochondria are thus less efficient in ATP generation, but are likely to produce damaging reactive oxygen species, and may tip the balance towards increased oxidative stress. Abnormal mitochondrial respiration was demonstrated in the gastrocnemius muscle biopsies of patients with PAD compared to controls using and oxygen sensing *Clark* electrode (Pipinos *et al.*, 2003). In a further gastrocnemius muscle biopsy study, Pipinos *et al*. measured the enzymatic activities of the mitochondrial oxidative phosphorylation (complexes I-IV), finding significant reductions in patients with intermittent claudication compared to healthy controls. Importantly, markers of oxidative stress, such as depleted antioxidant stores and evidence of oxidative damage, were also associated with the presence of PAD suggesting a possible link between defective mitochondrial function and oxidative stress (Pipinos *et al.*, 2006). Given that mitochondria are the primary production site of ROS, spill over may directly promote endothelial dysfunction and alter cellular function, specifically myofibres and mitochondrial DNA (Weiss *et al.*, 2013; Koutakis *et al.*, 2014). Indeed, the observation of

significantly elevated levels of apoptotic endothelial cells in gastrocnemius muscle biopsies of patients with claudication (Mitchell *et al.*, 2007) may be linked to the local overproduction of ROS. Interestingly, analysis of the DNA content of mitochondria from patients with claudication has shown the accumulation of multiple mutations which may also be due to ROS toxicity (Bhat *et al.*, 1999; Brass & Hiatt, 2000). Whether or not mutations in mitochondrial DNA are mechanistically linked to the symptoms or disability of claudication is unclear, but it could be suggested that mitochondrial mutations represent a consequence of high levels of oxidative stress in patients with PAD. It is an interesting finding that multiple mitochondrial mutations are also present in the muscle biopsies taken from the unaffected legs of patients with unilateral disease (Bhat *et al.*, 1999). One can speculate that this may reflect a high degree of generalised oxidative stress. Preliminary evidence suggests that some aspect of the metabolic myopathy, such as delayed phosphocreatine recovery that accompanies chronic peripheral ischaemia, can be reversed by successful revascularisation (West *et al.*, 2012). It would be important to known if other mitochondrial abnormalities can be reversed by exercise, revascularisation or medical therapy.

#### 2.3.4.4 **Muscle Fibre Types**

Skeletal muscles demonstrate heterogeneity in the constituent muscle fibres and a number of techniques are available to identify specific myofiber subtypes. The fundamental myofiber types can be discriminated into type I and type II fibres using histochemical techniques such as myosin ATPase or myosin heavy chain (MHC) staining. A particular muscle is constituted of a mixture of fibre types in which one type may predominate depending on the function of that muscle (see Table 2.3). Type I fibres are known as ‘slow-twitch’ fibres are high in aerobic

capacity due to an abundance of mitochondria and myoglobin and are therefore relatively resistant to fatigue, but they tend to be smaller and thus generate less contractile force. As such, type I muscle fibres are more abundant in postural muscles and those involved in long periods of activity such as the quadriceps and soleus muscles of the leg. In contrast, type II fibres are known as 'fast-twitch' fibres, have high glycolytic capacity, relatively lower aerobic capacity, fewer mitochondria and thus have relatively less resistance to fatigue. Type II fibres are relatively larger than type I fibres and are found in muscles which perform rapid and brief force generation such as extraocular muscles responsible for eye movement. Type II fibres can also be subdivided based on the presence or absence of oxidative ability with IIA fibres having a moderate oxidative capacity and type IIX having very little. In addition histochemical typing, muscle fibres can be characterised by the predominant isoform of the MHC expressed. Table X broadly summarises the characteristics of the fundamental muscle fibre types in humans.

Table 2.3. Characteristics of skeletal muscle fibre type

| | Type I | Type IIA | Type IIX |
|---|---|---|---|
| Other names | Slow oxidative | Fast oxidative /glycolytic | Fast glycolytic |
| Myosin ATPase activity | Slow | Fast | Fast |
| Diameter | Small | Large | Large |
| Glycolytic capacity | Moderate | High | High |
| Mitochondrial content | High | Moderate | Low |
| Oxidative Capacity | High | Moderate | Low |
| Predominate MHC isoform | MHC I | MHC IIa | MHC IIx |
| Force generation | Small | Medium | Large |
| Resistance to fatigue | High | Moderate | Low |

#### 2.3.4.5 **Microscopic Alterations in the Muscles of Patients with PAD**

Histological analysis of muscle biopsies taken from patients with claudication compared with healthy controls has shown a lower cross-sectional area of type I fibres, and smaller type II fibres in the symptomatic legs of patients with unilateral claudication (Clyne *et al.*, 1982, 1985). However, evidence of type II fibre atrophy has also been demonstrated in a gastrocnemius biopsy study by Regensteiner et al. who compared the results patients with claudication to healthy controls (Regensteiner *et al.*, 1993). A further study by Koutakis et al. observed that patients with claudication had significantly lower myofiber cross-sectional area compared to healthy controls, and that this was as strong predictor of walking

performance on the 6MWD test and maximum walking distance (Koutakis *et al.*, 2015). In a more recent muscle biopsy study a lower proportion of type I fibres and smaller fibre cross-sectional area was found in patients with claudication compared to controls (Askew *et al.*, 2005). Furthermore, type I fibre type cross sectional area was associated with walking performance on a graded treadmill test in patients, but not in healthy controls. Thus, type I fibres appear to be important in walking capacity. In the process of claudication, there is likely a shift in the muscular phenotype, away from oxygen dependent fibres type I fibres, toward rapidly fatigable glycolytic type II fibres. This makes intuitive sense as the underlying pathology is peripheral ischaemia.

Nerve dysfunction as also been investigated amongst patients with PAD and intermittent claudication. Nerve conduction studies have found evidence of peripheral neuropathy even at early stages of PAD. Koopman et al. found nine out of eleven patients with claudication studied had lower limb peripheral neuropathy (Koopman, de Vries & de Weerd, 1996). In a larger study, of 979 community-dwelling patients, (109 with PAD), peroneal nerve conduction velocity was significantly associated with PAD together with lower calf muscle cross-sectional area measured by computed tomography (McDermott *et al.*, 2004b). A further association of PAD with nerve dysfunction was identified in a large cross-sectional study of 770 patients (478 with PAD), particularly those with severe ischaemia defined by and ABPI of <0.50 (McDermott *et al.*, 2006). Again, similar findings were replicated by Garg *et al.* in a cross-sectional study of 668 patients (413 with PAD) finding impaired peroneal nerve conduction velocity and lower calf muscle cross-sectional area, but that this was associated with poorer walking performance on the 6MWD (Garg *et al.*, 2011).

In addition to impaired peripheral nerve function observed by nerve conduction studies, there has long been indirect microscopic evidence of nerve abnormalities in the leg muscles of patients with intermittent claudication. This was phenomenon was recognised as early as 1982 in the gastrocnemius muscle biopsies of patients with claudication that appeared to have high frequencies of small angular fibres, indicative of chronic denervation (Clyne *et al.*, 1982). Furthermore, type I and II muscle fibres are normally randomly distributed, but denervation of muscle fibres is followed by reinnervation by axons of an adjacent motor unit. Once reinnervated, the fibre type transforms to match all fibres in the motor unit. This results in fibre type grouping rather than the normal random distribution. Fibre type grouping has been observed frequently in the gastrocnemius biopsies of patients with claudication compared to healthy controls reinforcing the evidence of neuropathy and denervation (Clyne *et al.*, 1982; Regensteiner *et al.*, 1993). What causes denervation is not known, but one could speculate that repetitive ischaemia-reperfusion injury and high levels of ROS play a role. Nevertheless, denervation and atrophy are likely harmful consequences PAD and probably contribute to weakness, poor functional performance and sarcopaenia.

#### 2.3.4.6 **Macroscopic Alterations in the Muscles of Patients with PAD**

Sarcopaenia, which literally means “poverty of flesh” is generally accepted to mean the age relate decline in muscle mass and strength (Rosenberg, 1997). In the general population, after the age of 30 approximately 3-8% of muscle mass is lost every 10 years (Volpi, Nazemi & Fujita, 2004). This contributes to falls, age-related frailty syndromes, and results in the loss of independence for many elderly

people (Janssen, Heymsfield & Ross, 2002). Sarcopaenia affects about 20% of people over the age of 65 years and more than 50% aged over 85 years and is thus a significant public health problem (Iannuzzi-Sucich, Prestwood & Kenny, 2002). Sarcopaenia, however, can be accelerated by many disease states such as renal failure, chronic lung disease, cancer, as well as PAD (Buford *et al.*, 2010). For example, Addison *et al*. looked at a cross-section of 108 community dwelling men older than 50 years, of which forty-two had PAD confirmed on ABPI. They found that when appendicular lean muscle mass was quantified by DEXA, sarcopaenia was ten times more prevalent in patients with PAD compared to well matched control participants (10% vs. 1 %, p=0.004) (Addison *et al.*, 2018). Moreover, patients with both PAD and sarcopaenia walked about 14% less during a 6MWD test than PAD patients who did not have sarcopaenia, highlighting the association of poor muscle mass on walking ability. Other imaging studies that have used CT and MRI of the muscles of interest and found patients with claudication have less calf muscle cross-sectional area and lower density, when assessed by CT, which has been demonstrated to be independently associated with mobility loss and overall mortality (McDermott *et al.*, 2009b, 2012b). A more recent retrospective CT study a cohort of 223 patients with PAD found that leg muscle density, but not muscle volume, was independently associated with myocardial infarction, stroke, or cardiovascular death (Morris *et al.*, 2018). The authors point out that low muscle density may be a sign declining muscle strength, which may occur more rapidly than loss of volume, and possible reflect a change in the phenotype of muscle fibres given observations in muscle biopsy studies of patients with PAD.

In addition to lower muscle mass, patients with intermittent claudication have poorer lower limb strength compared to healthy controls (Regensteiner *et al.*,

1993) and lower limb strength, across hip, knee and ankle, is highly correlated with the haemodynamic severity measured by ABPI, even when adjusting for known confounders (McDermott *et al.*, 2004b; Parmenter *et al.*, 2013). It is not known if the observed changes in leg strength are due to direct consequences of ischaemia or are the result of disuse atrophy. However, the lowest ABPI is highly correlated with strength in both legs even in patients with significant differences in ABPI, suggesting an association with haemodynamics even if disuse atrophy plays a role (McDermott *et al.*, 2004a). These strength deficits are likely to affect quality of life and other complications. For instance, neck of femur fractures in the elderly is an injury that is intrinsically linked to falls and poor muscle strength (Nguyen *et al.*, 2005). Indeed, in among men over the age of 65 years, an ABPI <0.9 is strong independent predictor of subsequent hip fracture (HR = 1.69; 95 % CI, 1.08, 2.63 )(Hyde *et al.*, 2013). A recent meta-analysis of six studies, incorporating 233,835 patients (15,895 patients with PAD), also reported a significant association between PAD and hip fracture with the pooled RR of 1.64 (95% CI, 1.17–2.29) (Ungprasert *et al.*, 2018). In addition, lower limb strength in patients with PAD has been demonstrated to be associated with increased mortality (Morris *et al.*, 2014; McDermott *et al.*, 2012b).

Interestingly, sarcopaenia appears to be associated with endothelial dysfunction. In cross-sectional study of 595 apparently healthy middle aged Korean men those with the lowest appendicular skeletal muscle mass were significantly more likely to have highest carotid intima-media thickness, which is an additional marker of endothelial dysfunction (Heo *et al.*, 2018). In another Korean study of 236 community dwelling women over 40 years old, weaker hand-grip strength which is a surrogate for sarcopaenia, was significantly correlated with endothelial

dysfunction (Kim *et al.*, 2014). In octogenarians in Brazil, low skeletal muscle mass, but not fat mass, was associated with high coronary artery calcification and FMD (Campos *et al.*, 2017). Although these studies don't inform causation, it is possible that interventions, such as exercise therapy may result in improvement in either sarcopaenia, endothelial function both. It is interesting to speculate on mechanisms that may link exercise, muscle mass and to endothelial function and vascular inflammation. The emerging concept of small signalling molecules, released by working muscles, known as myokines and exerkines (exercised-induced myokines), may provide this link (Petersen & Pedersen, 2005; Pedersen & Febbraio, 2012; Yu, Tsai & Kuo, 2017).

In summary, patients with PAD and claudication tend to have weaker and lower quality muscles with evidence of a "chronic neuro-myopathy". Such changes are likely to be multifactorial with changes due the combined effects of local ischaemia and systemic factors such as oxidative stress and inflammatory burdens. Muscle changes resulting from exercise therapy are discussed in section 2.4.3.4.3.

### 2.3.5 **Role of Ischaemia-Reperfusion Injury**

Generally speaking, the extent of injury or cell death is dependent on the magnitude and duration of ischaemia as well as the tissue specific susceptibility. However, reperfusion can paradoxically lead to an increased damage to ischaemic but otherwise viable tissue, a concept first noted by Jennings *et al.* in reperfused hearts of dogs (Jennings *et al.*, 1960). Ischaemia at the cellular level results in a poverty of oxygen and energy substrates and thus decreased adenosine triphosphate (ATP) generation via oxidative phosphorylation. Creatine phosphate stores in skeletal muscle serve as a buffer by donating high energy phosphate to

adenosine diphosphate (ADP) (Cowled & Fitridge, 2011). However, as creatine phosphate stores deplete and ATP levels fall further, there is a shift towards inefficient anaerobic metabolism via glycolysis, which is accompanied by the build-up lactate and free hydrogen ions, lowering the pH (Pipinos & Casale, 2014). This intracellular acidosis decreases ATP generation even further by inhibiting glycolysis. In addition, ADP is further broken down into adenosine monophosphate (AMP) and inosine monophosphate (IMP), adenosine and hypoxanthine (Gillani *et al.*, 2011). With ongoing ischaemia and the depletion of cellular ATP, ionic pumps fail leading to the accumulation of intracellular sodium and calcium ions which leads to cellular oedema, damage to membranes and protein structures, as well as activation of cellular proteases such as calpain (Pipinos & Casale, 2014). Prolonged ischaemia results in irreversible injury and cell death via necrosis. Reperfusion is needed to resupply oxygen and energy substrates for oxidative phosphorylation, however resupply of oxygenated blood can promote the generation of reactive oxygen species resulting in peroxidation of cell membranes and thus increased membrane permeability, endothelial activation, and release of proinflammatory and prothrombotic mediators (Gillani *et al.*, 2011). When severe, reperfusion injury can exacerbate local ischaemic injury, and induce distant injury such as systemic inflammatory response syndrome and multi-organ failure (Kalogeris *et al.*, 2012). However, exposure to a moderate level or duration of ischaemia followed by reperfusion can trigger cellular dysfunction without cell death, exemplified by the phenomenon of myocardial stunning and hibernation which follow moderate bouts of ischaemia (Bolli & Marbán, 1999). In contrast, very short bouts of ischaemia have been shown to be protective of future prolonged ischaemic episodes in animals models (Murry, Jennings & Reimer, 1986; Schott *et al.*, 1990; Kalogeris *et al.*, 2012) and

humans (Speechly-Dick, Grover & Yellon, 1995). This concept know as ischaemic pre-conditioning is thought to activate survival adaptations (Kalogeris *et al.*, 2012). The mechanisms behind ischaemic preconditioning are incompletely understood, but are thought to be triggered when transient ischaemia results in the release of mediators such as adenosine, bradykinin and endogenous opioids, which in turn activate protein kinase C to activate second messengers, which inhibit mitochondrial transition pore formation and prevent cell death (Liu *et al.*, 1991; Goto *et al.*, 1995; Schultz *et al.*, 1995).

Patients with intermittent claudication experience muscle ischaemia during exercise followed by reperfusion at rest, and are thus chronically exposed to a low-grade form of repetitive ischaemia-reperfusion. In many ways, this is analogous to chronic exposure to ischaemic preconditioning, however, whether this results in a degree of ischaemic protection is not known. It is interesting to note that in animal models of induced myocardial infarction, that chronic hind-limb ischaemia reduces infarct size compared to no limb-ischaemia. Nevertheless, patients with chronic limb ischaemia who have a sudden deterioration in perfusion due to in situ thrombosis often have milder symptoms compared to patients who experience a sudden decrease in perfusion due to embolic disease (Earnshaw, 2014). Whilst this difference is widely believed to be the result of compensatory and possibly protective collateral development and remodelling in the case of chronic ischaemia (Earnshaw, 2014), it is possible that chronic exposure to ischaemia-reperfusion results in a beneficial ischaemic preconditioning adaptation. In support of this concept is that haemodynamic indices do not necessarily differentiate acute from acute on chronic ischaemia. Thus, it is conceivable the chronic cycles of ischaemia and reperfusion that are experienced

by the exercising patients with claudication may stimulate local ischaemic resistance and contribute to increases in walking ability seen after supervised exercise therapy.

Remote ischaemic preconditioning (RPIC) is also a concept worth considering. Remote ischaemic preconditioning is phenomenon where brief ischaemia-reperfusion in one region can result in ischaemic resistance at a site remote from the (Przyklenk *et al.*, 1993). There is an increasing number of studies that suggest close similarities between an exercise stimulus and remote ischaemic preconditioning. For example, an interesting study by Michelsen *et al*. demonstrated that dialysate collected from the blood of healthy volunteers after 8 minutes of vigorous interval exercise or 20 minutes of ischaemic preconditioning, significantly reduced the myocardial infarct size in isolated rabbit hearts (Michelsen *et al.*, 2012). Together this suggests there is a humoral factor that circulates in increasing levels after both RIPC and exercise that can confer resistance to a myocardial ischaemia-reperfusion injury, even across species (Michelsen *et al.*, 2012). In addition, neuronal reflexes within the autonomic nervous system have also been shown to be important in mediating the protective effects on the endothelium (Loukogeorgakis *et al.*, 2005). Remote ischaemic preconditioning may explain the benefits of exercise in healthy people and may indicate a global benefit of exercise in the claudicant population. Indeed, low level of physical activity is a risk factor for the development on PAD (Wilson *et al.*, 2011) and independently predicts cardiovascular events and mortality in retrospective and prospective cohort studies (Gardner, Montgomery & Parker, 2008; Garg *et al.*, 2006). On the other hand, the repetitive exposure to ischaemia experienced by patients with claudication is conceivably harmful if each episode

results in the accumulation of oxidative stress and inflammatory burden. Such a process could plausibly contribute to the excess cardiovascular morbidity and mortality observed in this patient group.

## 2.4 **Management of PAD**

The two main aims of the treatment of PAD are firstly to reduce the risk of subsequent cardiovascular events and death and secondly to reduce the disabling symptoms and thereby improve mobility and quality of life. Current treatment options include non-pharmacological lifestyle and exercise interventions, medications, and surgical and catheter-based endovascular treatments. The following is a brief review of the evidence and rationale for each.

### 2.4.1 **Risk Factor Modification**

#### 2.4.1.1 **Smoking Cessation**

Substantial evidence from observational studies has demonstrated reduced risk of death, cardiovascular events, and progression to critical limb ischaemia and amputation in patients who quit smoking (Jonason & Bergström, 1987). However, at least 2-4 years of abstinence are required to reduce the cardiovascular risks of smoking (Rosenberg, Palmer & Shapiro, 1990) and successful long-term cessation rates are extremely low when patients attempt to quit without the assistance from medical professionals (Law & Tang, 1995). Physician provided advice to cease smoking has been reported to approximately double the unassisted quite rate, of 2-3%, in a meta-analysis of 42 trials comprising over 31,000 smokers (Stead *et al.*, 2013). Pharmacologic therapy such as nicotine replacement therapy (NRT), buproprion and varenicline increase successful long-tern quit rates dramatically over placebo meta-analysis of randomised controlled trials (Cahill *et al.*, 2013, 2016) and this is reflected in latest AHA/ACC guidelines for the management of PAD (Gerhard-Herman *et al.*, 2017).

Recently non-tobacco nicotine inhalation devices have become widely available. Such devices are commonly known as vapers or electronic cigarettes and are filled

with a glycerine solution containing various concentrations of nicotine and other constituents such as flavourings. The vapours are thought to contain fewer harmful substances and at lower doses compared to the vast array of produced by tobacco combustion and inhalation. Therefore, use of such devices may be less harmful than traditional smoked tobacco. Preliminary evidence consisting of two RCTs involving over 600 participants suggests that e-cigarettes containing nicotine increase the chances of quitting compared to e-cigarettes without nicotine, without any serious adverse effects at two years follow-up (Hartmann-Boyce *et al.*, 2016). Whether e-cigarettes are more than NRT or other pharmacotherapies is unknown. Additionally, there is very little data on the long-term cardiovascular effects and other health impacts of e-cigarettes and regulation of sale and distribution such devices and liquids vary widely around the world. The trade of nicotine-containing e-cigarette fluids is currently illegal in Australia despite a recent application for an amendment to the Poisons Schedule (Joint Advisory Committee on Chemicals and Medicines Scheduling., 2017). More research in this area is needed before recommendations can be made.

#### 2.4.1.2 **Antiplatelet Therapy**

Antiplatelet therapy with aspirin is recommended for patients with PAD (Norgren *et al.*, 2007; Aboyans *et al.*, 2018; Gerhard-Herman *et al.*, 2017). Early support for the use of antiplatelet agents in PAD came from large meta-analyses of patients with coronary or cerebrovascular disease where subgroups analyses of patients with PAD were performed. The largest of these studies is the Antithrombotic Trialists Collaboration, which reviewed 287 RCTs that showed that antiplatelet therapy reduced the odds of death, stroke or myocardial infarction

by 23% overall (Antithrombotic Trialists' Collaboration, 2002). Despite aspirin being the most used agent in the overall study, for the PAD subgroup, a significant proportion of patients picotamide was the antiplatelet used, weakening the strength of the argument for aspirin. The CLIPS trial was the first multi-centre placebo controlled RCT of aspiring monotherapy designed specifically for patients with PAD. This study demonstrated a 64% risk reduction in major vascular events MI, stroke and pulmonary embolism at 2 years (Critical Leg Ischaemia Prevention Study (CLIPS) Group, 2007). Following the CLIPS, Berger *et al.* performed a more focused meta-analysis of aspirin compared to placebo in PAD in 2009 and demonstrated a significant reduction on non-fatal stroke and a 12% relative risk reduction in death, stroke, or MI, but the later did not reach statistical significance (Berger *et al.*, 2009). However, this meta-analysis has been criticised for lacking statistical power and generalisability since only two out of the 18 trials used doses of aspirin found most efficacious from Anti Thrombotic Trialists Collaboration meta-analysis (McDermott & Criqui, 2009). A recent meta-analysis by Mahmoud *et al.,* however, has again cast doubt on the efficacy of aspirin in PAD, reporting that based on 11 trials comprising 6,560 patients with PAD, aspirin did not alter major adverse cardiovascular events, myocardial infarction or intracranial haemorrhage (Mahmoud *et al.*, 2017).

Clopidogrel has also been evaluated to reduce the cardiovascular risk in PAD. The CAPRIE study randomized patients with cardiovascular disease, with a large PAD subgroup, to clopidogrel or aspirin and found a significant relative risk reduction of 23.8% amongst in favour of those who received clopidogrel in the PAD subgroup at 2-year follow-up (CAPRIE Steering Committee, 1996). Given its similar adverse events profile to aspirin, clopidogrel is considered a safe and

effective alternative to aspirin for risk reduction in patients with PAD receiving a Level I, A recommendation in the 2016 AHA/ACC guidelines (Gerhard-Herman *et al.*, 2017).

Building on this finding, and of the success of dual antiplatelets in acute coronary disease the use of combination clopidogrel and aspirin was evaluated in the CHARISMA study which randomised 15,603 patients with symptomatic cardiovascular disease or multiple risk factors to clopidogrel and aspirin or aspirin monotherapy. Overall, there was no significant difference in the primary composite of endpoint of myocardial infarction, stroke, or death from cardiovascular causes, but a trend towards increased moderate bleeding in patients on dual therapy (Bhatt *et al.*, 2006a). Subsequently a post hoc analysis of the PAD subgroup of 3,096 patients was performed and confirmed no benefit in terms of the composite endpoint (CV death, myocardial infarction, stroke), however a significant reduction in the rate of MI and hospitalisation for ischaemic events was observed in the dual antiplatelet group, with no significant difference in the rates of moderate, severe, or fatal bleeding (Cacoub *et al.*, 2009). Despite this evidence of a small effect, dual antiplatelet therapy is not currently recommended for the first line management of intermittent claudication (Aboyans *et al.*, 2018).

The recently published Examining Use of Ticagrelor in Peripheral Artery Disease (EUCLID) trial investigated whether ticagrelor monotherapy was superior to clopidogrel monotherapy in 13,885 patients with symptomatic PAD, with approximately 75% in each group reporting claudication. Following 30 months follow-up there was no significant difference in the primary efficacy endpoint (CV death, MI, stroke) or the primary safety endpoint, major bleeding (Hiatt *et al.*, 2017). Currently no guidelines have incorporated the result of this trial.

#### 2.4.1.3 **Dyslipidaemia Therapy**

As it is a risk factor of PAD, it is unsurprising that medical therapy for dyslipidaemia is recommended in patients with intermittent claudication. Like the evidence that supports the use of antiplatelet therapy, most of the evidence for lipid lowering therapy such as the statins (hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors), is derived from large scale RCTs of patients with coronary artery disease or multiple risk factors and not necessarily isolated PAD. For example, the landmark Scandinavian Simvastatin Survival Study (4S) randomised patients with CAD and high cholesterol to simvastatin or placebo and found significant reductions in all-cause mortality and coronary events (4S Study Group, 1994). Similarly, the Heart Protection Study (HPS) a large RCT of patients with established atherosclerotic disease (including 6,748 with PAD) or multiple risk factors, who were randomised to simvastatin or placebo, demonstrated a 13% reduction on all-cause mortality due mainly to a reduction in vascular death (Heart Protection Study Collaborative Group, 2002). Subsequent meta-analysis of large RCTs confirmed the benefit of statins in secondary prevention in patients with cardiovascular disease (Baigent *et al.*, 2005). Trials specific for patients with PAD have been less numerous, however analysis of the PAD subgroups from the above large trials have demonstrated benefit. For example, in the PAD subgroup of the HPS (n=6,748) there was a 6.3% absolute risk reduction of major vascular events, defined as major coronary events, strokes of any type, and coronary or non-coronary revascularisations (Heart Protection Study Collaborative Group, 2007). In addition to the effects on CV events and mortality, statins appear to also affect limb related symptoms. In a post-hoc analysis of the 4S study, simvastatin demonstrated significantly lower incidence of new or worsening claudication compared to placebo (Pedersen *et al.*, 1998).

Likewise, a case-control study McDermott showed a significant association between statin use and walking performance and limb function in patients with PAD after correcting for multiple potential confounders (McDermott *et al.*, 2003). In addition, data from a randomised controlled trial of patients with intermittent claudication, which compared low-dose (10mg), high-dose (80mg) atorvastatin and placebo demonstrated less vascular events in the statin groups, and improved pain-free walking distance in the high-dose group compared to the placebo group (Mohler *et al.*, 2003). More recently observational data from the REACH registry collaboration demonstrated an approximately 18% lower rate of adverse limb outcomes, including worsening symptoms, peripheral revascularization, and ischaemic amputations when statin use was compared to no statin use (Kumbhani *et al.*, 2014). Additionally, high intensity statin therapy has been observed to be associated with improved survival and fewer adverse cardiovascular events when compared to low or moderate intensity dosing (Foley *et al.*, 2017). Whilst the data supporting the prescription of statin therapy is quite clear, statin therapy tends to be underutilised in patients with PAD compared to those with CAD (Subherwal *et al.*, 2012) and similar to antiplatelet therapy efforts should be made to increase the appropriate prescription of this life saving medication.

Apart from lowering LDL levels, statins exert beneficial off-target effects on the endothelium and inflammatory mediators, such as improvement of endothelial dysfunction, increased nitric oxide bioavailability, antioxidant effects, inhibition of inflammation, and stabilization of atherosclerotic plaques (Davignon, 2004). Together, these pleiotropic effects of statins are likely beneficial given that atherosclerosis is a systemic inflammatory state.

In addition to statin therapy, a PCSK9 (proprotein convertase subtilisin/kexin type 9) inhibitor offer an alternative pathway to target LDL. One such PCSK9 inhibitor, evolocumab, has recently been evaluated in a large (n=27,564) multicentre randomised controlled trial comparing evolocumab to placebo in patients already on statin therapy (Hamilton, 2017). In the subgroup analysis of 3,642 patients with PAD (69.3% with current claudication), evolocumab significantly reduced the primary efficacy endpoint (CV death, MI, stroke, hospital admission for unstable angina or coronary revascularisation) at 2.5 years (HR 0.79, p=0.0098). This 6.3% absolute risk reduction translates in a number need to treat of 16 to prevent one major adverse cardiovascular or limb event. Whether evolocumab or other PCSK9 inhibitors gain recommendations for the treatment of PAD remain to be seen.

Current society guidelines recommend that all patients with PAD have their serum LDL lowered to <1.8mmol/L, or decreased by 50% where the initial level is 1.8-3.5mmol/L (Aboyans *et al.*, 2018; Conte *et al.*, 2015).

#### 2.4.1.4 **Anticoagulation Therapy**

The use of anticoagulants such as heparin, low-molecular weight heparin (LMWH), or oral anticoagulants such as warfarin has been assessed in a meta-analysis of seven randomised trials in patients with intermittent claudication (Cosmi, Conti & Coccheri, 2014). Overall, there was no significant effect on mortality or cardiovascular outcomes and no improvement in claudication symptoms. However, there was a significantly greater number of major and minor bleeding episodes with oral anticoagulants versus control which lead the authors

to conclude that there is no clear evidence to support the use of anticoagulants in intermittent claudication.

Recently reported results from an international multicentre double-blind, prospective randomised controlled COMPASS PAD trial may challenge this (Anand *et al.*, 2017). In this trial, there were n=7,470 patients with PAD were randomised to a combination of low dose aspirin (100mg daily) and rivaroxaban (2.5mg twice-daily), aspirin (100mg) alone or rivaroxaban alone (5mg twice-daily). Combination treatment compared to aspirin alone resulted in a 28% relative risk reduction of the primary composite endpoint of cardiovascular death, stroke or myocardial infarction (HR 0.72, 95% CI 0.57–0.90, p=0.0047) and 46% relative risk reduction in major adverse limb events (vascular intervention or major amputation due to a vascular cause (HR 0.54 95% CI 0.35–0.82, p=0.0037). This was offset by a 1.6% increase in the relative risk of major bleeding which was predominantly gastrointestinal in nature (p=0.0089). Importantly there was no statistically significant increase in fatal or non-fatal intracranial bleeding or bleeding into a critical organ with combination rivaroxaban and aspirin versus aspirin alone. Whether this large trial will translate into a change in practice guidelines remains to be seen.

#### 2.4.1.5 **Antihypertensive Therapy**

In addition to the above pharmacotherapies aimed at secondary risk prevention, antihypertensive therapy is recommended by the major society guidelines on PAD management to reduce the events of cardiovascular events. A target BP <140/90 mmHg is recommended for most patients with PAD, except those with diabetes or

renal failure, for whom the recommendation is <130/80mmHg (Norgren *et al.*, 2007; Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018). Similar to the evidence for the other therapeutic targets above, the majority of the evidence comes from large studies of patients with coronary artery disease or atherosclerotic disease in general rather than focused PAD trials. For example, the multicentre placebo controlled Heart Outcomes Prevention Evaluation (HOPE) trial (n= 9,297) showed a 22% reduction in vascular death, MI and stroke due to ramipril 10mg daily for 5 years (HR 0.78; 95% CI 0.70-0.86; p<0.001; NNT=27) (Yusuf *et al.*, 2000). In this trial, nearly half of the participants had PAD defined by an ABPI <0.9. The ABCD trial which randomised n=201 patients with diabetes and PAD to moderate or intensive blood pressure lowering and showed a significant reduction in vascular events, independent of drug class (Mehler *et al.*, 2003). Interestingly the benefit seemed greater in patients with lower ABPIs at baseline. The subsequent ONTARGET trial of n=25,620, which included over 13% with PAD, randomised to ramipril, telmisartan or both confirmed that telmisartan was non-inferior to ramipril in terms of the composite end point of cardiovascular mortality, MI, stroke or heart failure hospitalisation (RR 1.01, 95% CI 0.94-1.09, RR 0.99, 95% CI 0.92-1.07) (The ONTARGET Investigators, 2008). Shahin *et al.* conducted a small (n=33) randomised placebo controlled trial of ramipril in patients with claudication finding a significant improvement in treadmill walking distance (Shahin, Cockcroft & Chetter, 2013). Similarly, Zankl *et al.* performed a single centre, single blinded RCT of telmisartan versus placebo in 36 patients with claudication, finding that treadmill walking distance and flow mediated dilation (FMD) (Zankl *et al.*, 2010). In addition, there was a significant improvement in ABPI in non-diabetic patients. As supported by the two large trials above angiotensin converting enzyme inhibitors or angiotensin receptor blockers are

generally used first line in patients with PAD (Norgren *et al.*, 2007; Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018). However, not all patients can tolerate ACE-I with approximately 4.2-7.3% needing to discontinue due to cough (The ONTARGET Investigators, 2008; Yusuf *et al.*, 2000). Beta-adrenergic blocking drugs were initially speculated to worsen claudication symptoms, however, evidence of harm has not been supported by evidence from randomised trials and PAD guidelines have stated that this particular class of drugs can be safely used in patients with PAD, and probably has additional benefits in patients with ischaemic heart disease (Radack & Deck, 1991; Lane & Lip, 2013; Paravastu, Mendonca & Da Silva, 2013).

It has also been suggested that blood pressure lowering may worsen symptoms and leg ischaemia, however a recent meta-analysis of randomised placebo-controlled trials found no adverse outcome in terms of maximum walking distance, pain free walking distance or ABPI. In fact, the reduction in mean arterial pressure correlated with maximal walking distance (Manapurathe *et al.*, 2017).

#### 2.4.1.6 **Diabetic Therapy**

Large well conducted randomised controlled trials of different treatments have been performed in both the type 1 and type 2 diabetic populations. The Diabetes Control and Complications (DCCT) trial randomised type 1 diabetics to intensive versus conventional treatment. Intensive treatment consisted of insulin ≥3 times daily or via external pump; dosages adjusted according to self-monitoring of blood glucose four times per day; monthly clinic review and frequent phone calls; aiming for a monthly HbA1c <6.05%. In contrast, conventional treatment consisted of injections of insulin one or two times daily; self-monitoring of urine

or blood glucose daily, ± daily adjustments; clinic appointments every three months but no specific HbA1c target. The results demonstrated a significant reduction in the primary endpoints of retinopathy and nephropathy, but no difference in macrovascular complications or PAD endpoints (Diabetes Control and Complications Trial Research Group *et al.*, 1993) . After the conclusion of the trial, >96% of the participants were continued onto intensive treatment and were followed for a further 17 years. During this observational period, there was a 42% risk reduction in major cardiovascular events despite the fact that the HbA1c values between the original groups had converged, indicating a hangover of earlier poor glycaemic control on macrovascular complications (Nathan *et al.*, 2005). Very long follow-up data of the original cohort has shown that the beneficial effects of early intensive glycaemic control persist for up to 30 years (Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) Study Research Group, 2016).

In a similar way, the UKPDS 33 trial looked at intensive vs. conventional treatment in type 2 diabetics. Like the DCCT, there were highly significant reductions in microvascular complications during the 10-year follow-up, but no difference in mortality or MI. In the post-trial follow-up of a further 10 years, in which all patients were switched to intensive treatment, there was convergence of HbA1c, but a significant reduction in all-cause mortality and risk of MI. There was however, no significant difference in stroke or PAD outcomes such as amputation or death from PAD (UK Prospective Diabetes Study Group, 1998).

A number of other trials investigated whether very aggressive glycaemic control would be beneficial in terms of micro and macrovascular complications. The Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) and Veterans Affairs Diabetes Trial (VADT) trials

showed no benefit in macrovascular complications (The ADVANCE Collaborative Group, 2008; Duckworth *et al.*, 2009), whilst the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial (aiming HbA1c <6%) trial demonstrated a significant increase in the risk of hypoglycaemic episodes, cardiovascular death, and death from any cause (Action to Control Cardiovascular Risk in Diabetes Study Group *et al.*, 2008). Current guidelines on PAD recommend "strict" glycaemic control without a specific numerical target (Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018). However, it would seem safest to avoid unrealistic targets such as HbA1c <6% in patients with PAD.

It has previously been postulated that metformin might increase fibrinolytic activity and thus blood flow and endothelial function (Chakrabarti, Hocking & Fearnley, 1965; Fearnley, 1969; Laursen & Gormsen, 1969). Metformin has been previously hypothesised to improve functional capacity in patients with intermittent claudication. One small non-randomised study found that low dose (500mg twice daily) in 11 patients with claudication increased post-ischaemic hyperaemia, and also treadmill PFWD and maximal walking distance (MWD) (Montanari *et al.*, 1992). This, however, was not associated with significant reductions in total fibrinolytic activity. A prospective placebo-controlled randomised clinical trial was planned and registered to test whether a higher dose (1000mg twice daily) for 16-18 weeks could improve treadmill tested walking performance (Australian New Zealand Clinical Trials Registry, 2016). Unfortunately this trial was terminated prematurely due to difficulties with recruitment.

### 2.4.2 **Pharmacotherapy for Claudication Symptoms**

A number of agents which aim to improve symptoms and function in claudication have been assessed. These other agents do not fall into category of drugs which are aimed at managing traditional risk factors as described above.

#### 2.4.2.1 **Pentoxifylline**

Pentoxifylline (oxpentifylline) is a methylxanthine derivative and is a competitive non-selective phosphodiesterase inhibitor that causes the intracellular accumulation of the second messenger cAMP. Taken orally 2-3 times per day, it is approved for the treatment of intermittent claudication in the US, UK, Australia and New Zealand (National Institute for Health and Care Excellence, 2011; eTG Complete, 2018; NZ Formulary, 2018; Norgren *et al.*, 2007). Its mechanism of action in intermittent claudication is poorly understood, but it is thought to exert its therapeutic effect by decreasing blood viscosity, increasing erythrocyte deformability, inhibiting platelet aggregation, lowering plasma fibrinogen and promoting fibrinolysis and thereby improving microcirculatory blood flow and tissue oxygenation (McCarty, O'Keefe & DiNicolantonio, 2016). A recent pooled analysis of randomised controlled trials of pentoxifylline was unable to reach any conclusion due to a lack of well reported data despite 17 such studies being performed (Salhiyyah *et al.*, 2015) . Therefore, no clear recommendation can be made with respect to claudication and it is not recommended by the major society guidelines (National Institute for Health and Care Excellence, 2011; Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018).

#### 2.4.2.2 **Naftidrofuryl**

Naftidrofuryl (nafronyl) has been approved for the use in intermittent claudication for over 30 years, but only in the UK and European countries (Norgren *et al.*,

2007). Naftidrofuryl inhibits 5-hydroxytrptamine type 2 receptors and is thought to improve muscle metabolism, and reduce erythrocyte and platelet aggregation, but its exact mechanism in claudication is not defined (Barradell & Brogden, 1996). A meta-analysis of seven placebo controlled randomised trials, that included individual patient data on n=1,266 patients, found that naftidrofuryl resulted in a significant and clinical meaningful increase in pain-free walking distance by 37% (95% CI 27-49%, p<0.001), translating in a NNT of 4.5 (95% CI 3.6 to 5.8) (de Backer *et al.*, 2012). Based on network meta-analysis of trials, the NICE Technology Assessment Group found naftidrofuryl to have the greatest clinical effectiveness and acceptable safety cost effectiveness profile of available pharmacotherapies (National Institute for Health and Care Excellence, 2011).

#### 2.4.2.3 **Cilostazol**

Cilostazol is specific phosphodiesterase type III inhibitor that increases intracellular concentrations of cAMP, thereby causing vasodilation and inhibition of platelet aggregation. It is taken orally, twice per day. A Cochrane meta-analysis which included 15 double-blind, randomised controlled trials of n=3,718 participants indicated that cilostazol can improve maximum walking distances (100mg twice daily vs placebo, WMD 31.41 metres, 95% CI 22.38 to 40.45 metres; P < 0.00001), and uniquely, ABPI (Bedenis *et al.*, 2014). Although statistically significant, it is questionable whether this results in a clinically meaningful improvement and whether this would positively affect quality of life. Despite this, it is one of the two FDA approved drugs for claudication (the other pentoxifylline) and considered a reasonable therapeutic option in Australia by the Therapeutic Good Administration (eTG Complete, 2018). It is generally well

tolerated but its side effects which include oedema, headaches, and diarrhoea, can result in in discontinuation in up to 20% of patients (Hiatt, Money & Brass, 2008).

#### 2.4.2.4 **Nutritional Supplements**

A supplement rather than a targeted drug, carnitine has also been evaluated as a therapy to increase walking performance and the quality of life of patients with claudication. It has been shown that the ischaemic muscles of patients with claudication result in depletion of carnitine which is necessary for the transfer of energy intermediates across the mitochondrial membranes (Brevetti *et al.*, 1991) . Carnitine supplementation with acetyl-L-carnitine or propionyl-L carnitine in a range of difference preparations has been evaluated in a number of small pre-test/post-test, parallel and cross-over randomised clinical studies, with most finding significant but small increases in walking performance (Delaney *et al.*, 2013).

One multicentre double-blind randomised placebo-controlled clinical trial found that heparan sulphate (40mg orally twice a day) improved PFWD to a greater extent than placebo and fewer patients worsened during the trial follow-up (Messa & Gelso, 2002).

The recent Resveratrol to Improve Outcomes in Older People With PAD (RESTORE) trial found no evidence that resveratrol, a sirtuin activator found in red wine, improved walking performance (McDermott *et al.*, 2017b).

There is also insufficient evidence to recommend other proposed treatments such as metformin, buflomedil, prostanoids, Ginko biloba extract, Padma 28, vitamin E and omega-3 fatty acids (Kleijnen & Mackerras, 1998; Campbell, Price & Hiatt, 2013; de Backer & Vander Stichele, 2013; Robertson & Andras, 2013; Nicolaï *et al.*, 2013).

### 2.4.3 **Exercise Therapy**

#### 2.4.3.1 **General Benefits of Exercise**

Regular physical activity is widely accepted and recommended as a primary prevention strategy against cardiovascular disease and premature mortality with risk of all cause and cardiovascular mortality inversely related to physical activity (Paffenbarger Jr. *et al.*, 1993; Winzer, Woitek & Linke, 2018). The WHO recommends adults aged 18–64 to perform: (1) at least 150 minutes of moderate-intensity aerobic physical activity throughout the week or do at least 75 minutes of vigorous-intensity aerobic physical activity throughout the week or an equivalent combination of moderate- and vigorous-intensity activity, and (2) muscle-strengthening activities 2 or more days a week (World Health Organization, 2015b). Similar recommendations are made for adults over 65 years old (World Health Organization, 2015a).

In large unselected adult population studies, physical activity even below the WHO recommendations, compared to relative inactivity, has been found to be beneficial in terms of reducing all-cause mortality (Wen *et al.*, 2011). Increased physical activity, compared to relative inactivity, exerts a number of positive effects reducing the risks cancer, cardiovascular and metabolic disease, depression

and anxiety, osteoporosis and physical dependence in later life (Warburton, Nicol & Bredin, 2006; Aylett, Small & Bower, 2018). When compared to drugs and invasive interventions, exercise is irrefutably lower cost and relatively free of adverse effects.

#### 2.4.3.2 **Exercise as an Intervention in PAD**

Furthermore, the majority of patients with intermittent claudication do not meet recommended standards for physical activity (Lauret *et al.*, 2014b). As a treatment strategy for intermittent claudication, exercise therapy has long been believed to benefit patients with intermittent claudication and was first suggested by Erb in 1898 (Erb, 1898). Larsen conducted the first randomised controlled trial in 1966 (Larsen & Lassen, 1966) and over the last 50 years there– has been a substantial amount of investigation into the optimal form of exercise and its mechanism of action. Many of the studies have small numbers and only a fraction of the hundreds of studies are well designed prospective randomised controlled trials. Much of the investigation has considered the effects of supervised treadmill training 2-3 times per week for periods raging of 6-52 weeks, but some have included other forms of exercise. Weight training and resistance exercise has been evaluated, as have the effects of supervision versus non-supervision, which will be discussed further below. Nearly all studies of supervised exercise have resulted in positive trend or significant improvement results in terms of walking performance. A systematic review and meta-analysis by Lane *et al*., incorporating 30 trials and a total 1,816 participants has summarised the benefit of exercise for claudication (Lane *et al.*, 2017). This analysis included trials of treadmill walking, Nordic pole-striding, upper and lower aerobic exercise, and resistance training and were

supervised sessions twice per week. Overall, pain free walking times increased in the range of 3-6 minutes when patients were assessed on a treadmill. Similarly, pain-free walking distance increased in the range of 72-93 meters and improvements were seen in up to two years. Based on these results the authors concluded that exercise programs in general are of significant benefit in walking performance when compared to placebo, but were inconclusive with regard to mortality and limb loss (Lane *et al.*, 2017). However, it is of paramount importance to note that the included studies were not designed nor powered to detect changes in mortality and limb loss. In addition, these conclusions are not without other limitations. Many of the studies were small and used inherently different methods for assessing outcome measures such as graded or constant load treadmill or overground walking tests. This becomes problematic when considering the pooled data for the purpose of meta-analysis.

The limitations of treadmill walking tests have been excellently summarised (McDermott *et al.*, 2014) and include the points: (1) that treadmill walking tests are not representative of walking in daily life (Greig *et al.*, 1993), (2) a clinically meaningful improvement in treadmill walking has not been defined in claudication, and (3) there is a significant learning effect associated with treadmill walking evident by patients who improve walking performance in the placebo arms of RCTs (Hiatt *et al.*, 2010; Creager *et al.*, 2011). Furthermore, since most of the trials used treadmill based primary outcome measures, there is potential bias towards finding positive outcome in those who had the opportunity to practice and become familiar with walking on a treadmill. McDermott *et al*. refers to this issue as the "training to the outcome advantage", which is unable to be ignored when considering the results of trials where the intervention is nearly identical to the

primary outcome measure (McDermott *et al.*, 2014). Additionally, it is known that hand-rail support during treadmill walking increases both pain-free walking distance and maximal walking distance (see Figure 2.12)(Gardner, Skinner & Smith, 1991). Conversely, it has been observed that six-minute overground walk tests more closely correlate with outdoor walking capacity and health-related quality of life, than does a graded treadmill test (Nordanstig *et al.*, 2014a). As Nordanstig *et al*. point out, this is probably due to the fact that real life walking is generally at a slower pace and longer duration than that which occurs during a treadmill test. Furthermore, there are definite biomechanical differences between treadmill and overground. For example, it has been observed that hip movements and gluteal muscle activation are significantly greater during treadmill walking compared to overground walking (Lee & Hidler, 2008; Anders *et al.*, 2019). The external validity of such a test is therefore limited. The learning effects and other peculiarities of treadmill training and testing must be also considered, especially when studying trial data that compares a treadmill-based exercise program to any other intervention, when the primary outcome measure is treadmill-walking. Calls for standardisation of outcome measure terminology has been made and future research should adhere to this (Stoner *et al.*, 2016) but there remains considerable debate about the role of the treadmill in assessing clinical efficacy of exercise therapy (McDermott *et al.*, 2014; Hiatt, Rogers & Brass, 2014) . This aside, it seems that the ability of supervised exercise to improve walking performance is no longer in question.

#### 2.4.3.2.1 **Safety and Long Term Outcomes of Supervised Exercise Therapy**

Safety is aspect of supervised exercise programs that has received little attention in the literature with only one study focusing on this. Gommans *et al*. performed a

systematic review of trials of supervised exercise and obtained data on complications and expressed them as events per number of patient hours (Gommans *et al.*, 2015). Seventy-four studies comprising 2,876 patients met the inclusion criteria. Only eight adverse events were recorded resulting in an all-cause complication rate of one per 10,340 hours of exercise. Whilst this may appear to demonstrate an impressive safety record, it is important to note that only 35 of 74 studies explicitly reported the possible occurrence of adverse events. No mention was made of whether Gommans *et al.* contacted the authors of the included studies to ascertain the incidence of unreported adverse events. In addition, many studies excluded patients with limited exercise capacity due to angina, cardiac failure and chronic obstructive pulmonary disease. As such this may represent a significant underestimation of the true adverse event rate of supervised exercise and may lack external validity as many real-world patients have these conditions.

The long-term outcomes, particularly limb morbidity and mortality are still poorly addressed in the literature. One prospective randomised controlled trial of 264 patients with claudication, which compared open surgical revascularisation, to supervised exercise or control, reported one year amputation and mortality of 2.2% and 5.6% respectively (Gelin *et al.*, 2001). The authors noted that there was no significant difference between the groups and the rates were in line with epidemiological data available at the time. One study of Japanese patients with intermittent claudication (Fontaine II) retrospectively analysed the long-term cardiovascular morbidity and mortality outcomes depending if patients completed 12-weeks of supervised treadmill walking (3 days per week), or not (Sakamoto *et al.*, 2009). One hundred and eighteen patients were enrolled in the supervised

treadmill-based exercise program, of which 64 patients completed it, compared to 54 patients who did not. The baseline characteristics did not differ between the completers and non-completers in terms of age, comorbidities, ABPI and medical therapy. Maximum walking distance at baseline was significantly greater in completers (475+/- 369 m vs. 332.8 +/- 251 m, p=0.018). In multivariate analysis, however, an interaction between maximum walking distance at baseline and completion of exercise training program was not revealed. Using Cox proportional hazards regression analysis, over the median follow-up period of 5.7 years, those who were able to complete a supervised exercise program were significantly less likely to suffer a cardiovascular death (HR 0.16, 95% CI 0.05-0.54, p=0.003). Obviously, this study is limited by the fact that it was a retrospective non-randomised analysis. Some authors have raised concerns about the lack of data regarding cardiovascular outcomes in patients undergoing supervised exercise. In trying to answer this question of the effect of supervised exercise on cardiovascular outcomes, they have investigated the effect of supervised exercise on factors that can be linked such as muscle wasting, inflammatory response and endothelial function. These studies and their results are reviewed and discussed in Section 2.4.3.4.

One recent study has questioned the long-term durability of supervised treadmill waking specifically in terms of the overground walking in the 6-minute walk test endpoint (McDermott *et al.*, 2019). In this study, 156 patients with intermittent claudication were randomised to 6-months of supervised treadmill walking, resistance training, or a non-exercising attention control group. At 6-month mark, the supervised group improved the 6MWD, compared with control (+36.1 m, 95% CI +13.9- 58.3, p=0.001). However in the 6-months following this, the 6MWD

declined significantly such that at 12-months there was no significant difference in 6MWD between the supervised group and the control group. McDermott *et al.* reasonably postulate that poor durability of supervised treadmill exercise may be due to the fact that: (1) it has less potent effect on 6MWD than overground home-based exercise because it is not relevant to walking during daily life, and (2) treadmill exercise is associated with a learning effect that may decay with time in the absence of continued practice, and (3) the testing the 6MWD is not associated with a learning effect.

#### 2.4.3.3 **Different forms of Exercise Therapy and Walking Performance**

A discussed above, a number of exercise interventions to improve the symptoms of intermittent claudication and walking performance have been investigated. Notwithstanding the limitations already described, most of these studies have been conducted on treadmill walking since they allow control of other walking variables such as speed, incline, duration, and are often located in the stable environment of a gymnasium. In addition, treadmill walking enables large groups of patients to be supervised and motivated by trained staff members. Other forms of exercise investigated include pole striding, cycling and upper or lower limb ergometry, resistance training, or combination training, which can be supervised or unsupervised.

##### 2.4.3.3.1 **Walking versus Other Forms of Exercise**

Non-treadmill based forms of training have been evaluated in a number of studies, for example Jones *et al.* randomised patients with claudication to either treadmill training or StairMaster training over 12 weeks, finding that both improved

treadmill walking performance, but more so in the treadmill trained group (Jones *et al.*, 1996). Interestingly, those in the StairMaster group improved their StairMaster performance more so than those in the treadmill trained group suggesting a "training to outcome" learning or familiarisation effect as described above in (Section 2.4.3.2). Supervised upper- or lower-limb ergometer training performed twice per week for 6 weeks improved pain-free walking distance and maximal walking distance in an overground shuttle-walk test compared to non-exercising controls (Walker *et al.*, 2000). A randomised trial from the same group showed that a 24-week program of upper- versus lower- limb exercises produced similar significant improvements in overground walking in a shuttle-walk test (Zwierska *et al.*, 2005). It is likely that the positive effect on of upper limb ergometry results from an improvement in general aerobic fitness which is probably one of the factors in determining walking capacity. Similar findings were noted by Treat-Jacobson *et al.* who randomised patients to 12 weeks of supervised upper-limb ergometer training or treadmill walking, finding no difference in the improvement in walking performance (Treat-Jacobson, Bronas & Leon, 2009). In contrast to the studies above by Walker and Zwierska *et al*., Sanderson *et al*., found no overall improvement in a graded treadmill test outcome, in patients with claudication randomised to 6-weeks of supervised cycle-training versus supervised treadmill training (Sanderson *et al.*, 2006). This is surprising given the near identical nature of cycling and lower-limb ergometer training, however may be due to the relatively short duration of the intervention. Nordic pole walking, in which patients use specially designed light-weight walking poles to support the upper body during walking, has been shown to immediately improve treadmill assessed walking performance after familiarisation (Oakley *et al.*, 2008). Furthermore, patients randomised to 12 weeks of minimally

supervised Nordic pole walking significantly improved treadmill assessed walking performance compared to non-exercise control (Langbein *et al.*, 2002). A more recent study demonstrated a superior effect of Nordic pole walking compared to home-based exercise (Spafford, Oakley & Beard, 2014).

A systematic review and meta-analysis of randomised controlled trials was performed by Lauret *et al*. to investigate the effects of different forms of exercise on walking performance in intermittent claudication (Lauret *et al.*, 2014a). A total of 5 studies incorporating 135 participants that included supervised walking compared to other regimens such as cycling, strength or upper limb ergometry were pooled for analysis. Interestingly, pole walking was not considered an alternative exercise to supervised walking and was not included in this analysis. This concept was confirmed recently in a systematic review, where it was demonstrated that Nordic pole-walking offered no advantage over standard walking in terms of walking performance (Golledge *et al.*, 2018a). Nonetheless, with the meta-analysis on different forms of exercise, differences in outcome measure used, limit the ability to pool data from different studies. However, the authors elegantly converted distances and walking times into metabolic equivalents using the validated American College of Sports Medicine walking equation which takes into account walking speed and grade (Glass & Dwyer, 2007). When this was done, there was no clear superiority of supervised walking over the combined results of other forms of exercise in terms of pain free walking distance and maximal walking distance nor when walking exercise was compared to strength training or combination training. A limitation of this meta-analysis is that the conversion of walking performance assessed by other methods such as an overground shuttle-walk cannot easily be converted to METs and were therefore

excluded from the pooled data analysis. Furthermore, biomechanical differences between treadmill walking and overground walking may be significant and confounding. Nevertheless, a recent randomised controlled trial of different exercise regimens, which have used non-treadmill based assessment of walking performance, have found results not dissimilar to the above meta-analysis (Delaney *et al.*, 2014; McDermott *et al.*, 2009a). These studies support the conclusion that no specific mode of supervised exercise has yet been found to be superior in terms of walking performance.

Figure 2.12. The supervised exercise environment at the Repatriation General Hospital gymnasium. Notice all patients naturally prefer to hold onto the hand rails which is dissimilar to overground walking.

#### 2.4.3.3.2 **Supervised versus Unsupervised Exercise**

Barriers to the implementation of supervised exercise therapy, such as lack of accessible and convenient facilities and non-funded status (Popplewell &

Bradbury, 2014) have hindered their wide spread adoption and motivated the investigation of non-supervised exercise as an acceptable alternative. On this front, the US seems to be leading the way globally with the recent announcement that the Centers for Medicare and Medicaid Services (CMS) will provide financial coverage for SEP in patients with claudication (Jewell, Shishehbor & Walsworth, 2017). Coverage provides for 36 sessions over 12-weeks, in a supervised setting similar to current society guidelines. Despite this progress, it is likely that many will find attending a supervised exercise program burdensome, even when it made available for free (Harwood *et al.*, 2016). They are additionally inconvenient for patients who are employed and cannot attend due to work commitments. A recent study reported that as many as 69% of eligible patients with claudication refused participation in supervised exercise (Harwood *et al.*, 2016). Furthermore, a recent survey of patients with claudication reported that the majority of patients would prefer home-based exercise over a hospital-based SEP (Harwood *et al.*, 2018). Difficulty in getting patients walking is compounded by lack of widespread access to SEP, an issue highlighted in a survey of vascular surgeons in the UK, which showed that only 24% had access to supervised exercise program and only 14% recommended 100% of eligible patients (Shalhoub, Hamish & Davies, 2009). A similar international survey of mainly European vascular surgeons revealed only about 30% had access to a supervised exercise program, highlighting the underutilisation of this intervention (Makris *et al.*, 2012). To address this, there has been interest in utilising home-based walking exercise as an alternative to supervised exercise. A number of randomised trials have compared supervised exercise training versus non-supervised training. For instance, Meyer *et al*. randomised 20 patients with claudication to 12 weeks of supervised or unsupervised exercise (advice to walk with weekly contact for compliance and

encouragement), finding no improvement in treadmill assessed walking performance outcomes in the unsupervised group (Meyer *et al.*, 1997). Limitations of this study include the small sample size and the possibility of making a type II error, and the likelihood that those in the supervised group were able to practice and become accustomed to walking on a treadmill. In contrast, Patterson *et al*. randomised 55 patients to supervised vs home-based exercise (weekly exercise instruction) and assessed the outcomes after a 12-week period. Pain-free walking time and maximal walking time improved in both groups and were sustained at 6 months, however between group differences favoured those in the supervised group assessed by a progressive treadmill test (Patterson *et al.*, 1997). In 2013, Fokkenrood *et al*. published a systematic review and meta-analysis of randomised controlled trials of supervised versus non-supervised exercise therapy (Fokkenrood *et al.*, 2013). For the included trials, non-supervised programs were defined by advice to walk or could consist or structured home-based exercise without direct supervision. Fourteen studies comprising 1,002 participants were included with follow-up ranging from 6-52 weeks. All trials used a treadmill walking test as an outcome measure and supervised training was superior to non-supervised exercise leading the authors to conclude that vascular health professionals should make supervised exercise therapy available to all patients with intermittent claudication. However, it is worth bearing in mind that those who underwent supervised training almost all did so on the treadmill, whereas few if any in the non-supervised group had access to treadmill exercise. This fact, together with the known learning effect of treadmill experience, likely biases overall walking performance outcome in favour of those in a supervised group.

In the Group Oriented Arterial Leg Study (GOALS), McDermott *et al*. randomised 194 patients with claudication to weekly group mediated cognitive behavioural therapy designed to help participants adhere to home-based walking exercise or an attention control group (McDermott *et al.*, 2012a). At six months, the intervention group had significantly improved both overground 6-minute walk distance and maximal treadmill walk time, whereas the control group did not (mean difference, 53.5 m [95% CI, 33.2 to 73.8]; P <0 .001) (McDermott *et al.*, 2013c). Importantly, the effect of CBT and home-based walking exercise program remained durable out to 12-months follow-up even though the CBT sessions had finished at 6-months. In another a trial by Gardner *et al*., 180 patients were randomised to 12 weeks of supervised treadmill exercise, home-based step-monitored walking, and attention-control groups (Gardner *et al.*, 2014). Both supervised group and the home-based group improved treadmill-assessed pain-free walking distance and maximum walking distance, when compared to control. Interestingly though, when assessed in the overground 6MWT, the home-based group significantly outperformed the supervised treadmill group with a mean difference of 30m ($p<0.05$). This suggests that an overground walking training may be superior at improving walking performance when assessed by the overground 6-minute walk test. This is probably the case because training stimulus of home based walking more closely replicates the biomechanics of the overground 6MWT in comparison to the treadmill based testing. Further, total exercise time, measured by an ankle step monitor, was on average 300 minutes greater in the home-based group ($p=0.009$), suggesting that patients may have increased self-motivation when freed from the constraints of supervised sessions. Interestingly the lack of direct supervision in the home-based group did not result in a significant difference in the intensity of training, indirectly inferred by the

total volume of exercise in metabolic equivalent minutes, nor was there a significant difference in attrition, being approximately 20% in both exercise groups.

A recently reported meta-analysis of trials comparing structured home-based exercise versus no exercise program reported that reported that the former led to significant improvements in maximum walking distance, PFWD and 6MWD (Golledge *et al.*, 2019). Thus, structured home-based exercise appears to be clinically effective and may be cheaper, more convenient and result in higher participation compared to supervised exercise.

In summary, it appears that SEP of walking provides the clearest improvement in walking-performance especially when assessed using a treadmill based test. This fact is reflected in the Society consensus guidelines TASC II and NICE that recommends a supervised exercise all people with intermittent claudication (National Institue for Health and Care Excellence, 2012; Norgren *et al.*, 2007) . The recent 2017 ESC/ESVS guidelines on the management of PAD recommend preferably supervised exercise therapy giving a level 1A recommendation and a level 1C recommendation for unsupervised exercise when supervised training is not feasible or available (Aboyans *et al.*, 2018). Similarly, the latest 2016 AHA/ACC Guidelines on the management of PAD give a Level IA recommendation for structured supervised exercise to improve functional status and reduce leg symptoms. The AHA/ACC guidelines, however, go further and provide a IIa/A recommendation for structured community or home-based programs. The 2015 SVS Guidelines on management of asymptomatic PAD/claudication recommend SEP first-line 30-60 minutes for at least 12-weeks,

or home based if SEP is unavailable (Conte *et al.*, 2015). The above recommendations from the specialty societies is echoed by the recent decision by the US CMS to fund 12-weeks of supervised treadmill exercise. Despite this uncertainty remains in regard to the durability of supervised

#### 2.4.3.4 **Biological Effects of Supervised Exercise**

##### 2.4.3.4.1 **Changes in Haemodynamics and Perfusion**

Since the pathophysiology of claudication is intrinsically linked to muscle hypoperfusion it is tempting to suppose that the improvements in walking performance that follow exercise training are due to improvements in blood flow to the extremity either through increased collateral supply (macrovascular) or capillarisation (microvascular). Animal model data seem to support this notion, with exercise trained rats increasing distal limb perfusion after formation of a femoral stenosis (Mathien & Terjung, 1990; Yang, Ogilvie & Terjung, 1995). In support of this concept in humans is evidence that healthy subjects can improve capillary density after endurance training (Denis *et al.*, 1986). Such a response to exercise has been shown to be associated with VEGF expression and appears to be preserved in aging (Gavin *et al.*, 2007). However, Wang *et al.* failed to demonstrate a significant change in calf muscle capillarisation following 12 weeks of supervised exercise in eleven patients of similar age with intermittent claudication despite a significant increase in walking performance (Wang *et al.*, 2009). The authors instead reported a trend toward increased capillary contact with fast twitch glycolytic type II muscle fibres that correlated with the improvements in pain free walking time. On the other hand, another study of 35 patients with claudication who participated in 12 weeks of exercise training

demonstrated a significant increase in capillary density that was followed by an increase in peak oxygen uptake (Duscha *et al.*, 2011). A subsequent study by the same group again showed an increased capillary density following 12 weeks of supervised exercise training, but paradoxically this was associated with a decrease in muscle levels of proangiogenic VEGF-A and an increase in the VEGF165 inhibitor (Jones *et al.*, 2012). This surprising result is in contrast to the effects in healthy subjects and those with heart failure (Gustafsson *et al.*, 1999, 2001). This finding is in contradistinction to above study by Gavin *et al*. and may indicate a dysfunctional VEGF regulation in the setting of PAD (Jones *et al.*, 2012). Further investigation is needed into the regulation of microvascular changes that accompany improved walking performance.

On the macrovascular level, animal models of exercise training in the setting of arterial insufficiency have demonstrated an increase in hind limb blood flow due to collateral vessel enlargement (Prior *et al.*, 2004). In a human cross-sectional study, patients with superficial femoral artery occlusions with more numerous collateral vessels were associated with better 6-minute walk performance compared to those with fewer collaterals (p trend = 0.0002) (McDermott *et al.*, 2013b). However, in a study of 303 patients with PAD, those with larger or more numerous collaterals, were significantly more likely to be symptomatic and have lower ABPI values (Keeling *et al.*, 2012), suggesting that size and number of collateral vessels may be a marker of a more advanced stage of disease rather, than a beneficial adaptation.

To investigate the effects of exercise on blood flow various human studies have measured physiological markers such as ABPI, peak calf blood flow using venous

occlusion plethysmography, toe pressures, or transcutaneous oxygen tension. Parmenter *et al*. systematically reviewed the literature and found that among the 33 included trials incorporating 1,237 participants, there was no evidence of a significant increase in resting or post-exercise ABPI, nor reactive hyperaemic calf blood flow (Parmenter, Raymond & Fiatarone Singh, 2010). A subsequent Cochrane meta-analysis also failed any significant change in peak exercise calf blood flow (4 trials) or a clinically significant change in ABPI (13 trials) (Lane *et al.*, 2017). Consistent with the above findings, Versluis *et al.* used contrast-enhanced magnetic resonance angiography (CE-MRA) and showed no change in peak popliteal artery blood flow following 6 months of supervised exercise therapy despite a significant 71% improvement in pain-free walking distance (Versluis *et al.*, 2013). Importantly, the number of collaterals measured by CE-MRA did not significantly change following the exercise program implying that there was no macrovascular adaptation. Although this was a small study and has not yet been replicated, it adds weight to the argument that exercise training for intermittent claudication does not alter the lower limb macrovascular structure or function.

#### 2.4.3.4.2 **Endothelial Changes**

As mentioned above in the section of pathophysiology, the endothelium is key in the development of atherosclerosis and arterial insufficiency. It is thus unsurprising improving endothelial dysfunction has become a therapeutic target. A number of interventions such as statin therapy, angiotensin-converting enzyme inhibitors, and correction of hyperglycaemia have been shown to effective in correcting endothelial dysfunction (Davignon & Ganz, 2004). Exercise as a therapy to improve endothelial function has also attracted attention and it has been observed that physically active young adults have greater endothelial function

than sedentary (Pahkala *et al.*, 2011). Exercise induced increases in cardiac output and thus shear-stress, provide a plausible mechanistic link to the improvement of endothelial function (Wang, Wolin & Hintze, 1993; Clarkson *et al.*, 1999). Interestingly, exercise probably results in an acute deterioration in endothelial function that is followed by a return to baseline or supranormal level in a biphasic fashion (Dawson *et al.*, 2013). For instance, among healthy adults, moderate- and high-intensity exercise has been demonstrated to acutely decrease FMD when measured immediately after, which is then followed by return to baseline by 1 hour (Birk *et al.*, 2013). Furthermore, exercise training has been shown to improve brachial FMD in healthy individuals and patients with atherosclerotic risk factors (Ades *et al.*, 2011; Cornelissen *et al.*, 2013; Early *et al.*, 2017). The majority of data comes from studies that utilised predominantly aerobic endurance exercises, but also some which used resistance and high intensity interval training (Currie, McKelvie & MacDonald, 2012; Francois *et al.*, 2016). Similar improvements have been observed in patients with established asymptomatic coronary artery disease, where partial reversal of the abnormal response to intracoronary acetylcholine was observed following four weeks of exercise training, but not in non-exercising controls (Hambrecht *et al.*, 2000).

In patients with peripheral artery disease, the data is sparse and somewhat conflicting. Similar to studies of healthy adults described above, acute bouts of maximally tolerated walking exercise in patients with claudication have been shown to have a significantly reduced FMD with return to baseline values during recovery, in contrast to normal controls who had no significant change in FMD after 10min of walking exercise (Joras & Poredoš, 2008). In another clinical trial, acute treadmill exercise in patients with claudication caused a significant drop in

brachial FMD, that was attenuated in the group receiving an intravenous administration of L-propionyl carnitine, but not in the group receiving intravenous placebo (Silvestro *et al.*, 2006). The ability of L-propionyl carnitine to prevent exercise induced endothelial dysfunction may be to its antioxidant anti-inflammatory effects(Reznick *et al.*, 1992; Garrelds *et al.*, 1994). In addition, Andreozzi *et al*. demonstrated that the FMD acutely deteriorated following walking exercise among patients with claudication, however it was also shown that 6 weeks of supervised exercise training significantly improved the mean baseline FMD from 7.6% (2.94) to 10.3% (4.04) and the lessened the post exercise drop in FMD from -33.25 to -18.97% ($p<0.001$) (Andreozzi *et al.*, 2007a). Firstly, this suggests that exercise acutely results in a drop in endothelial function through the release of proinflammatory cytokines. In the long-term, however, it may trigger a positive adaptation process leading to improved baseline endothelial function with blunting of the negative impacts of acute exercise. Supporting this notion are several studies that assessed FMD before and after a chronic period of exercise training. For instance, Brendle *et al.* measured brachial FMD in 19 patients with claudication after 6 months of supervised treadmill exercise, noting a 60% increase in the resting values (Brendle *et al.*, 2001). Allen *et al*. conducted a RCT in 2010 randomising 15 patients with claudication to supervised treadmill training and 18 to usual care control, finding that FMD increased by +79% within the treadmill trained group (Allen *et al.*, 2010). Similar findings were reported by Mika *et al*. who reported an average 36% and 56% increase in brachial FMD in 55 patients with claudication who were randomised to 12-weeks of either pain-free or moderated pain-inducing treadmill exercise respectively (Mika *et al.*, 2013). Januszek *et al*. reported an average 42% improvement in brachial FMD after a 12-week program of supervised treadmill

walking (Januszek *et al.*, 2014). In contrast, McDermott *et al*. reported a non-significant improvement in brachial FMD in 37 patients with claudication randomised to 24-weeks of supervised treadmill exercise (0.70% (-0.77,1.82 p=NS) (McDermott *et al.*, 2009a). When compared to 28 controls who displayed a non-significant increase in brachial FMD, the between group difference just reached significance +1.53% (p=0.02). The reason for such a large difference in the results is unclear and is surprising since the treadmill training was very similar between studies. However, differing cohort demographics may explain this discrepancy since participants in the study by McDermott *et al*. were on average five years older, had twice the rate of diabetes mellitus, and were more likely to be morbidly obese. This is an important consideration since these factors are known to negatively affect FMD and the response to exercise (Henry *et al.*, 2004; Williams *et al.*, 2005; Black *et al.*, 2009). A recent randomised controlled trial with participants similar to the above trial by McDermott *et al*. that compared 12 weeks of treadmill exercise versus combination exercise failed to detect a change in brachial flow mediated dilatation (Delaney *et al.*, 2015). See Table 2.4 for summary of studies.

An attempt at summarising the data on the effect of supervised exercise on FMD was made in a systematic review and meta-analysis of randomised controlled trials by Parmenter *et al.* reporting that, from 9 clinical trials, there was no significant difference in ΔFMD between exercise and control groups (Parmenter, Dieberg & Smart, 2014). However, this meta-analysis pooled data from seven studies that measured resting and peak calf blood flow using venous occlusion plethysmography, one that measured brachial FMD, and one that didn't include a measure of non-invasive endothelial function at all. Thus, although these are different measures of endothelial function, it is questionable to pool data from

inherently different measurement techniques given that the technique is known to impact result.

Table 2.4. Studies investigating the effect of supervised exercise training on FMD.

| Study | Country | Study Type | Sample size | Training type | Duration of SEP | Cuff Position | Image Analysis | Result | Results |
|---|---|---|---|---|---|---|---|---|---|
| (Brendle *et al.*, 2001) | USA | OBS | 19 | 1. Near maximal treadmill walking | 6 months | Proximal | Manual | Positive | +60% (4.81 ± 0.82% to 7.97 ± 1.03% (p <0.005) |
| (Andreozzi *et al.*, 2007a) | Italy | OBS | 22 | 1. Treadmill walking to 60-70% PFWD | 6 weeks | Proximal | Manual | Positive | +35% (7.6 ± 2.94% to 10.3 ± 4.04% (p <0.001) |
| (McDermott *et al.*, 2009a) | USA | RCT | 156 | 1. Near maximal pain SEP<br>2. Lower limb resistance SEP<br>3. Control | 24 weeks | Proximal | Manual | Negative | 1. +12% (5.54 to 5.39, within group (0.7 (-0.77-1.82) p>0.05). Compared to control group +1.53%(.35-2.7, p=0.02)<br>2. +2.2% (4.89 to 6.13 within group 0.11 (-2.03-1.33) compared to control 0.9 (-0.58-2.37 p=0.23) |
| (Allen *et al.*, 2010) | USA | RCT | 35 | 1. Mod-severe pain SEP<br>2. Home-based exercise. | 3 months | Distal | Manual | Positive | 1. +56% (2.4% (0.08) to 4.3% (0.6) p<0.05.<br>2. +41% (0.5) to 3.2% (0.8), p>0.05. |
| (Mika *et al.*, 2013) | Poland | RCT | 60 | 1. Pain-free SEP<br>2. Moderate pain SEP | 3 months | Distal | Manual | Positive | 1. +56% (4.59 ± 2.14% to 6.27 ± 2.8% (p<0.01))<br>2. +36%, (3.98 ± 1.9% to 6.22 ± 2% (p<0.001)) |
| (Januszek *et al.*, 2014) | Poland | OBS | 67 | 1. Treadmill to moderate claudication pain | 12 weeks | Distal | Manual | Positive | Pre: +41.7% (4.34±2.37% to 6.15±2.4%, p<0.001)<br>Post: +43% (4.38±2.29% to 6.26±2.62%, p<0.0001) |
| (Delaney *et al.*, 2015) | Australia | RCT | 35 | 1. Treadmill SEP.<br>2. Combined SEP | 3 months | Distal | Automated | Negative | 1. Pre-FMD: 1.3 % (0.7-3.0) to post-FMD 1.3% (0.9-4.6) p=0.28.<br>2. Pre-FMD: 2.4% (0.4-5.0) to post-FMD 1.2% (0.1-4.4) p=0.11 |
| (Januszek *et al.*, 2016) | Poland | OBS | 59 | 1. Treadmill SEP to mild pain | 12 weeks | Distal | Manual | Positive | 1. Pre-FMD:+45% (4.16±2.26 to 6.05±2.34, p<0.0001)<br>2. Post-FMD: +42% (4.31±2.1 to 6.13±2.46, p<0.0001) |
| (Novaković *et al.*, 2019) | Poland | RCT | 29 | 1. Moderate-pain exercise<br>2. Pain-free exercise<br>3. Usual care | 12 weeks | Distal | Manual | Positive | 1. 4.4% (2.0) to 8.0% (2.3) p=0.002<br>2. 4.6% (1.6) to 6.9% (3.3) p>0.05<br>3. 5.9% (3.3) to 4.8% (2.0) p>0.05 |

Whether supervised exercise programs in claudication improve brachial FMD is clearly unsettled, thus further clinical data and a properly conducted meta-analysis of studies might usefully summarise the data to date. In doing so, it would be important to consider some of the technical factors that can affect the measurement of FMD. However, a number of barriers to completion of a successful meta-analysis exist such as the small number of randomised controlled trials, risk of bias, and heterogeneous outcome measurement protocol. In addition, the variable presentation of summary outcome data, often presented as median and interquartile ranges, is not easily incorporated into meta-analysis statistical packages unless sample sizes are sufficiently large to allow their substitution for mean and SD (Hozo, Djulbegovic & Hozo, 2005). An individual patient data meta-analysis may provide a solution to some of these limitations.

#### 2.4.3.4.3 **Muscle Changes**

As outlined above in Section 2.3.4, patients with claudication have characteristic changes in muscle function and structure that is associated with the disability. One important change is the accumulation of carnitine metabolites that results acetyl-CoA formation (see Section 2.3.4.3). Early clinical studies showed that treadmill based supervised exercise training can decrease the muscular and plasma build-up of acylcarnitine concentrations that is characteristic of untrained patients with claudication (Hiatt *et al.*, 1992, 1996). In addition, a small uncontrolled pilot study investigated the effect of supervised treadmill training using near-infrared spectroscopy to measure the recovery of oxygen consumption after exercise using this as marker of mitochondrial (Brizendine *et al.*, 2014). Using this methodology, the authors found that recovery of oxygen consumption, and thus mitochondrial function, improved significantly after 12 weeks of supervised exercise training.

Similarly, another recently published study assessed mitochondrial function, noting that positive responders to 8 weeks of walking and calf raising exercise also increased levels of mitochondrial respiration capacity and citrate synthase activity, whereas negative responders demonstrated decreased mitochondrial function (Schaardenburgh *et al.*, 2017). In addition, a number of studies have investigated muscle fibre phenotype following exercise. Hiatt *et al*. showed that supervised treadmill exercise training did not seem to alter the proportions of type I or type II fibre area in histological analysis despite causing the greatest improvement walking capacity (Hiatt *et al.*, 1996). Interestingly, despite treadmill training resulting in a mean 131% ($p<0.05$) improvement in pain-free walking distance, this group of patients also had evidence of a 105% ($p<0.05$) increase in number of denervated angular myofibres, whereas no such change was observed in resistance trained or control groups. This significance of this finding does not seem to have been investigated further and it is unknown whether this is a positive or negative effect of exercise therapy. More recent work by Beckitt *et al*. used a more sensitive Western blot technique to quantify myosin heavy chain isoforms and demonstrated an increase in MHC I, which is the predominant isoform found in the slow-twitch and most efficient oxidative muscle fibres. In patients following supervised exercise training, a 11% increase in MHC I expression occurred that did not occur in a non-exercising control group. Furthermore, this increase correlated with the improvement in PFWD on a fixed -grade treadmill test (Beckitt *et al.*, 2012).

With regard to muscle strength, Hiatt *et al*. showed that resistance training significantly increased muscle strength, whereas treadmill-trained and control patients experience no significant change (Hiatt *et al.*, 1994). In contrast, Wang *et al*. showed that supervised treadmill walking improved calf muscle strength and

torques (Wang *et al.*, 2006). The reason for this different result is not immediately clear but may relate to differences in the strength assessment methodology. A further interesting finding by Hiatt *et al*. was the strength gains in the resistance training group were lost when participants cross over and completed 12 weeks of treadmill training, suggesting that treadmill training does little to preserve muscle strength (Hiatt *et al.*, 1994).

A number of cross-sectional studies have linked reduced muscle size and quality with increasing severity of PAD, however, very few studies have investigated the effects of supervised exercise on these outcomes. Delaney *et al.*, proposed that the ischaemia-reperfusion exposure from supervised treadmill training would be associated with increased intracellular calcium concentration, and proteolytic enzyme (calpain) activation leading to cell death and muscle atrophy (Delaney *et al.*, 2014). To test this hypothesis, 35 patients with calf claudication were randomised to 12 weeks of treadmill-training or combination strength and treadmill training. Despite treadmill training resulting in a significant increase in calpain activity in biopsy from the symptomatic calf (+56%, p=0.05), there was no significant change in lower limb skeletal muscle mass of symptomatic whole lower limb (-1.0%, p=0.27), but small increase in muscle mass of the asymptomatic lower limb (+3.7%, p=0.03). On the face of it, it seems that treadmill-training might be associated with deleterious calpain activation and a relative loss of muscle mass or at least inhibited muscle growth. However, two important limitations of this study must be considered. Firstly, muscle mass analysis was performed by DEXA and included the entire lower limb as a whole, not just the symptomatic calf region. Thus, changes in muscle mass cannot be solely attributable to calf since it is impossible to tell if muscle mass of the thigh or buttock changed following interventions. In addition, muscle biopsies were

taken from the symptomatic calf muscle only, thus little can be said about calpain activation levels on the asymptomatic side or in the more proximal thigh musculature. Based on the above design, it would be flawed to link calpain activity in a calf biopsy with whole lower limb skeletal muscle mass. In addition, of the 35 patients randomised, there were only two patients included in the analysis of asymptomatic limbs thus this significant result may be due to a type I error. To date, no other studies have assessed the effects of supervised exercise therapy on skeletal muscle mass.

In summary, despite much interest in muscle changes following supervised exercise training for claudication, there are still large gaps in literature.

#### 2.4.3.4.4 **Immune and Inflammatory Changes**

The activities of cells of the innate immune system, such as neutrophils, have been studied in patients with PAD. In patients with claudication, it has been shown that exercise is associated with a decrease in neutrophil deformability, neutrophil degranulation and increased vascular permeability which may contribute to ischaemia through microvascular plugging (Hickey *et al.*, 1993; Khaira *et al.*, 1995). In addition, patients with mild claudication who complete exhaustive exercise to their maximal walking distance experience a significant rise in neutrophil activation and show signs of neutrophil elastase release which has been shown to be directly toxic to endothelial basement membrane (Turton *et al.*, 1998). Such activation has been thought to be a potentially deleterious systemic inflammatory response syndrome due to exposure to ischaemia-reperfusion in patients with intermittent claudication. Such a concept is supported by the observation that successful revascularisation surgery abolishes the

exercise-induced inflammatory response following exercise (Hickey *et al.*, 1990). This led some to hypothesise that exercise training in the presence of uncorrected haemodynamic insufficiency may be harmful by repetitive stimulation of an inflammatory response. To test this hypothesis, Tisi *et al*. conducted a prospective randomised controlled trial of exercise training versus observation, finding that while vascular permeability was increased following acute lower-limb exercise, 3-6 months of exercise training attenuated the acute post-exercise increase in vascular permeability and inflammatory markers (Tisi *et al.*, 1997). More recently Turton *et al*. showed that at rest patients with claudication and aged matched controls had similar levels of neutrophil activation and degranulation, however immediately post exercise, patients with claudication increased these levels whereas controls did not (Turton *et al.*, 2002). Furthermore, this response was attenuated in patients with claudication after completion of an intensive 3-month supervised exercise program, suggesting that training can decreased the injurious effects of ischaemia-reperfusion driven inflammation that occurs during claudication (Turton *et al.*, 2002). Conversely, two studies have investigated the inflammatory effects of upper-limb exercise in comparison to lower limb exercise or control. Nawaz *et al*. performed a randomised controlled trial and observed that lower-limb exercise caused an acute increase in neutrophil expression of CD11b and CD66b (both markers of neutrophil activation), that was not observed following upper-limb exercise (Nawaz *et al.*, 2001). After six weeks of training in either group, again there was no change in CD11b/CD66b expression after acute upper-limb exercise, however, neither training regimen was able to abolish the acute increase in expression following lower limb exercise, despite similar improvements in walking capacity. Together this suggests that gains in walking performance can be achieved via an upper-limb training strategy that avoids

neutrophil activation, but that effect of ischaemia reperfusion during walking persists. Saxton *et al*. also performed a randomised controlled trial with patients allocated to supervised leg-cranking, arm-cranking, or usual care control for 24 weeks and measured the concentrations of proinflammatory soluble adhesion molecule (VCAM-1, ICAM-1, E-selectin) and hs-CRP (Saxton *et al.*, 2008). None of the groups demonstrated a significant change in any of the markers at 24 weeks, however there was a non-significant tread toward a reduction in hs-CRP in both the arm-cranking and leg-cranking groups (p=0.07), which is possibly the result of a type II error because the study was not powered to detect a change in hs-CRP. Another study by Schagler *et al*. randomised patients with claudication to medical therapy and supervised exercise or medical therapy alone and failed to show any change in any of the classic markers of the inflammatory response, hs-CRP, IL-6, soluble P-selectin or monocyte platelet aggregates (Schlager *et al.*, 2012).

With regard to circulating monocytes, a recent study by Dopheide *et al*., followed 40 patients with intermittent claudication who underwent 12 months of home-based exercise and measured the proportions of classical, intermediate and non-classical monocytes. They found a significant increase in the proportion of classical monocytes with a corresponding significant decrease in the presumably proinflammatory and proatherogenic, intermediate and non-classical monocyte subtypes (Dopheide *et al.*, 2015). In addition, there were also reductions in neutrophil adhesion molecule (CD11b, CD11c) expression, which are both markers of neutrophil activation. This is the first study to demonstrate the association between exercise in patients with intermittent claudication and a decline in intermediate and non-classical monocyte subtypes. A lack of a

supervised exercise arm or control group is an important limitation of this study. Nevertheless, the findings are in line with observations from Timmerman *et al*. on the effect of exercise on monocyte subtypes in healthy but physically inactive people (Timmerman *et al.*, 2008). Together this suggests that exercise might be useful in reducing the inflammatory burden driven by both neutrophils and monocyte and macrophage.

### 2.4.4 **Surgical and Endovascular Intervention**

Since the pathophysiology of intermittent claudication is intrinsically linked to ischaemia thus arterial insufficiency, it is reasonable to hypothesise that correction of a haemodynamically significant arterial lesion would improve symptoms and function. Revascularisation is technically possible in most patients and can be achieved by traditional open surgical approach or via an endovascular approach. Open surgical approaches tend to consist of bypass procedures, whereas endovascular approaches mainly involve percutaneous transluminal balloon angioplasty, with or without stent insertion.

Early research on revascularisation focused on surgical revascularisation. Almost 30 years ago Lundgren *et al*. published a randomised clinical trial of surgical reconstruction alone (n=25) versus physical training alone (n=25) , versus surgical reconstruction and physical training (n=25) (Lundgren *et al.*, 1989a). Surgical reconstruction resulted in a significant increase in calf blood flow and ABPI, that was not observed in physical training group. Despite this, all treatments were effective at improving PFWD and maximum walking distance, however, surgery was more effective than physical training. The largest improvement in PFWD, however, occurred in the combination surgery and training. It is worth noting that

severe complications were higher among patients undergoing surgery; with three patient requiring haematoma evacuation, three requiring thrombectomy, and three requiring redo reconstructive surgery. In addition, one patient suffered a pulmonary embolism, and another died from an unrelated aortic dissection. In comparison, there were no deaths in the training group, however two patients developed limb-threatening ischaemia and two more suffered severe cardiac insufficiency and were unable to receive training. The high rate of complications and the improvement seen with exercise alone has led many to conclude that surgical revascularisation is unnecessary and high risk for the management of intermittent claudication (Phillips, Cowan & Johnson, 1997). However, some argue that provided surgical bypass can be constructed with minimal risks, it is a suitable alternative in patients with severe life-style or employment limiting symptoms that have failed conservative treatment (Whitehill, 1997).

With the rapid progression of endovascular technologies and increased enthusiasm, more contemporary studies compared supervised exercise with percutaneous transluminal angioplasty.

In Edinburgh, Whyman *et al*. screened 600 patients with intermittent claudication who had lesions that were deemed suitable for angioplasty (discrete stenoses or occlusions <10cm in the iliac or femoropopliteal arteries) (Whyman *et al.*, 1996). Only 62 (10%) of this group were then randomised to either angioplasty or medical therapy alone (low dose aspirin and advice on smoking and exercise). All patients were advised to continue to walk as frequently and as far as possible within the limits of pain. At six-months follow-up, significantly more patients in the angioplasty group were asymptomatic and also had significantly improved ABPIs. Additionally, PFWD and maximal walking distance was significantly greater in the angioplasty group compared to the medical therapy group leading

the author to conclude that angioplasty is superior to medical therapy alone. At 2-years follow-up, however there was no significant difference in treadmill claudication distance, maximum walking distance or ABPI (Whyman *et al.*, 1997).

In a pilot study conducted in Oxford, Creasy *et al*. randomised 36 patients with stable unilateral lower-limb claudication to angioplasty versus supervised lower-limb exercise finding that only angioplasty improved ABPI significantly (Creasy *et al.*, 1990). However, angioplasty only improved claudication distance at 3 months, and did not appear to improve maximal walking distance. In contrast, supervised exercise resulted in progressive improvements in pain free walking distance and mean maximal walking distance at three, six and nine-month follow-up, despite no improvement in mean ABPI. The complete long-term results of this study were later published in 1996 (Perkins *et al.*, 1996). At 15-months, claudication and maximal walking distance was still significantly better in the exercise group compared to the angioplasty group, however at a median of 6-year follow-up there was no significant difference between the groups. In addition, although this study was not powered for this outcome, there was no difference in overall mortality between the groups with four deaths in the angioplasty group and six in the exercise group, and there were two amputations in the entire study group.

Gelin *et al*. conducted a randomised trial of 264 patients with claudication allocating them to 6 months of supervised walking exercise therapy, revascularisation (open or endovascular), or observation (Gelin *et al.*, 2001). A total of 27 (21 open, 6 angioplasty) suprainguinal revascularisations, and 34 (23 open, 11 angioplasty) infrainguinal procedures were performed. Fifteen patients

randomised to revascularisation, did not complete treatment due to technical or medical grounds. In contradiction to the trial by Perkins *et al*, invasive treatment resulted in significantly increased (+22.6%, 274m (172) to 344m (169), $p<0.01$) maximal treadmill walking distance compared to supervised exercise (+4.35%, 258m (142) to 247m (111), p=NS) , which was no different to untreated controls on intention-to-treat analysis at 1 year. In addition, primary patency at 1 year was 89% for suprainguinal procedures and 76% for infrainguinal procedures. Importantly, there was no significant difference in mortality (approximately 5%) or major amputation at 1 year.

Subsequently, the Exercise versus Angioplasty in Claudication Trial (EXACT) randomised controlled trial was designed to compare balloon angioplasty with supervised exercise (Hobbs & Bradbury, 2006). Unfortunately, this study was terminated due to extremely poor recruitment, due to a number of factors such as presence of diffuse disease not optimally amenable to angioplasty, equal bilateral symptoms and clear preferences among patients as to whether they desired exercise therapy or percutaneous angioplasty.

The Adjuvant Benefit of Angioplasty in Patients with Mild to Moderate Intermittent Claudication (MIMIC) trial was conducted during 2003-2006, with the aim of determining if randomisation to adjuvant angioplasty conferred a benefit over supervised exercise therapy alone in patients with stable claudication due to either aortoiliac disease or femoropopliteal disease (Greenhalgh *et al.*, 2008). Sample size calculation indicated that 170 participants would be needed in each anatomical category ($\beta$ 90%, $\alpha$ 5%) to detect 60m improvement in absolute walking distance. Unfortunately, recruitment was stopped early, and only 34 and 93 patients were able to be randomised into the aortoiliac and femoropopliteal arms of trial, respectively. Despite this, there were significant improvements in

absolute treadmill walking distance conferred by adjuvant angioplasty in aortoiliac disease (+78%, p=0.05) and femoropopliteal disease (+38%, p=0.04). The Oslo Balloon Angioplasty versus Conservative Treatment (OBACT) trial randomised 56 patients to optimal medical therapy including home-based exercise advise alone, or with adjuvant angioplasty (Nylænde *et al.*, 2007). Patients had to meet strict inclusion criterial including having a lesion feasible for angioplasty evaluated by angiography. Over the 2-year follow-up, significant improvements in ABPI, pain free walking distance and maximum walking distance were observed in the group receiving adjuvant balloon angioplasty ($p<0.01$ for all) suggesting that adjuvant balloon angioplasty has a positive effect on haemodynamic and functional outcome. Patency data was not reported.

Spronk *et al*. conducted the single-centre Comparing Exercise Therapy with Angioplasty for Claudication (CETAC) trial, by randomising 151 patients with claudication to either revascularisation (via an angioplasty first approach), or to a hospital-based supervised treadmill-exercise program (Spronk *et al.*, 2009). Randomised patients had a combination of iliac and femoropopliteal disease that was not significantly different between study arms. In the adjusted intention-to-treat analysis, supervised exercise was significantly superior to endovascular revascularisation at 6-months in terms of pain-free walking distance (adjusted mean difference -16, -32 to 2, p=0.02), however no difference was observed at 12-months in either pain-free or maximum walking distance. Patency data was not reported. In the long-term follow-up at a median of 7 years, both supervised exercise and revascularisation resulted in significantly improved pain-free and maximum walking distances, however there was no statistically significant difference between the treatment groups (Fakhry *et al.*, 2013). There was no significant difference in cumulative survival probability (HR 1·35, 95% CI 0·67

to 2·70; p = 0.4). In addition, a significant number of patients allocated to the exercise group went on to have endovascular revascularisation after 1 year, however at the end of the long-term follow-up period, the total number of invasive procedures was nearly double in the original revascularisation group ($p<0.001$).

In the often-cited Claudication: exercise versus endoluminal revascularization (CLEVER) trial, 111 patients with aortoiliac occlusive disease were randomised to either medical care, optimal medical care plus 6 months of supervised treadmill walking, or medical care plus stent revascularisation (Murphy *et al.*, 2012). All patients were prescribed cilostazol, which was adhered to in >90% of patients in all groups. At six-month follow-up, supervised exercise resulted in the greatest increase peak-walking time, followed by stent revascularisation, then optimal medical care ($p<0.05$ for all). There was one myocardial infarction in the medical group, one death in the supervised exercise group and one target-lesion revascularisation in the stent group. In the stent group, there was one vessel perforation treated with a stent-graft and transfusion in the same patient, and two patients with localised dissections. At 18-months follow-up, both supervised exercise and stent insertion resulted in significantly improved peak walking time compared to medical therapy. However, there was no significant difference between supervised exercise and stent insertion suggesting that both are effective and superior to medical therapy alone, but the risks of invasive treatment can be avoided (Murphy *et al.*, 2015).

In the Invasive Revascularization or Not in Intermittent Claudication (IRONIC) Trial, Nordanstig *et al*. randomised 158 patients with claudication to an invasive or non-invasive treatment groups (Nordanstig *et al.*, 2014b). Participants in both

groups received information regarding structured training and including the use of Nordic walking poles. In addition, the invasive group underwent endovascular revascularisation for TASC II A-C femoropopliteal or iliac lesions, and open revascularisation for D lesions. At 12-months, both groups significantly improved their pain-free and maximum treadmill walking distances, however only pain-free treadmill walking distance was significantly improved (+124 (SD 196) vs. +50 (SD 99) m; p=0.003), favouring revascularisation. At 2-year follow-up the results were durable (Nordanstig *et al.*, 2016). In addition, 44% of patients in the invasive group reported they had fully achieved their treatment goals compared to only 10% in the non-invasive group.

Lindgren *et al*. recently published the results of a randomised control trial comparing primary stenting of TASC A-C SFA lesions to best medical therapy (without SEP) in 100 patients with stable intermittent claudication (Lindgren *et al.*, 2017). After 12 months follow-up, health-related quality of life, ABPI and treadmill assessed walking distance improved significantly in those undergoing stenting versus controls, with no significant difference in serious adverse events.

In contrast to the above studies, Marzari *et al*. focused on patients with claudication with femoropopliteal lesions suitable for angioplasty and randomised 178 patients to 12-weeks of supervised non-treadmill circuit exercise alone, angioplasty alone, or both (Mazari *et al.*, 2010). There were no complications in any of the treatment groups. Patients in all treatment arms significantly improved treadmill pain-free and maximum walking distance at 3-months, however the improvement was significantly larger in patients who received supervised exercise and revascularisation. In the long-term follow-up at a median of 5.2 years, there

was no longer any difference in treadmill walking distances, despite preserved improvement in the ABPIs in patients who underwent angioplasty or exercise and angioplasty, suggesting equal effectiveness (Mazari *et al.*, 2017). Interestingly, the addition of supervised exercise to angioplasty seemed to reduce symptomatic restenosis and reintervention rates.

The multi-centre Endovascular Revascularization And Supervised Exercise (ERASE) trial was conducted in Netherlands between 2010-2013 (Fakhry *et al.*, 2015). In this study, 212 patients, with intermittent claudication due to aortoiliac or femoropopliteal disease that was amenable to endovascular revascularisation, were randomised to 3-months of supervised treadmill exercise alone, or with endovascular revascularisation (with selective stenting). At 1-year follow-up, both groups experienced significant improvement in both pain-free and maximum treadmill walking distance, however, patients randomised to the combination exercise and revascularisation group, had a significantly greater improvement in these outcomes compared to exercise therapy alone ($p<0.001$).

A recently published meta-analysis included a total of 987 patients from seven of the trials discussed above (Spronk *et al.*, 2008; Perkins *et al.*, 1996; Gelin *et al.*, 2001; Greenhalgh *et al.*, 2008; Murphy *et al.*, 2012; Fakhry *et al.*, 2015) is the most extensive and contemporary summary of trials comparing endovascular revascularisation alone or in combination with supervised exercise therapy (Pandey *et al.*, 2017). Revascularisation alone was associated with significant improvement in ABPI compared to exercise therapy alone, however this was not associated with a significant improvement in treadmill-assessed pain-free or

maximum walking capacity, or risk of future revascularisation or amputation compared to supervised exercise therapy. In contrast, combination revascularisation and supervised exercise therapy resulted in a significant improvement in maximum treadmill walking distance compared to supervised exercise therapy alone (4 studies, 3 favouring combination therapy). A recent report confirmed the overall results of the meta-analysis (Klaphake *et al.*, 2018). Taken together this suggests that although both treatments can be effective, invasive revascularisation comes with significant risks which cannot be justified for equivalent gains that can be achieved by supervised exercise therapy alone. Thus, endovascular therapy alone is probably equivalent to supervised exercise therapy alone, however the former is likely to produce clinical effect quicker with less time commitment, albeit with attendant procedural risks. However, it seems that a combination of endovascular revascularisation and supervised treadmill exercise is currently the most efficacious.

#### 2.4.4.1 **Long Term Risks of Revascularisation**

Whilst revascularisation for intermittent claudication can offer the improved claudication symptoms, the risks and benefits of intervening must be weighed up. A recently published retrospective of 456 patients treated for intermittent claudication by two Australian hospitals has provided some insight to these risks (Golledge *et al.*, 2018b). The study cohort was stratified based on whether they underwent early revascularisation (<6 months from assessment) or initial conservative management (no revascularisation <6 months) and followed for a mean of 5-years. The 5-year risk of major amputation was approximately 5% in patients undergoing early revascularisation versus <1% in patients undergoing initial conservative management. Moreover, this risk was maintained even after

correcting for risk factors (including ABPI and baseline risk factors) in Cox proportional hazard analyses. In addition, the 5-year risk of secondary revascularisation was approximately 50% in patients undergoing early intervention, highlighting the fact revascularisation for claudication is not a once off procedure, but will likely result in the need for ongoing intervention.

With the high-risk of reintervention, it is not uncommon for interventionalists to use drug-eluting technology at secondary procedures to reduce the rate of restenosis. Currently available therapies include paclitaxel-coated angioplasty balloons and paclitaxel-eluting stents. Whilst these treatments have been demonstrated to significantly reduce restenosis in the superficial femoral artery, the long-term effects on mortality have not traditionally been a focus. A recent meta-analysis randomised trials of paclitaxel angioplasty and stent therapies for PAD, however, has reported a concerning finding of increased late mortality in patients receiving paclitaxel (14.7% versus 8.1% crude risk of death; RR, 1.93; 95% CI, 1.27–2.93) at four to five years (Katsanos *et al.*, 2018). This unexpected signal of increased mortality with paclitaxel endovascular therapy requires further investigation and the results of a large patient-level data meta-analysis is awaited.

#### 2.4.4.2 **Experimental Therapies**

Other novel interventions for intermittent claudication are being explored. Vascular endothelial growth factor (VEGF) gene therapy was evaluated in a phase II randomised controlled trial in patients with unilateral exercise-limiting claudication. Among the 105 patients randomised, the VEGF gene therapy treatment (VEGF-encoding replication deficient adenovirus) had no measurable effect compared to placebo, and was associated with an increase in peripheral

oedema (Rajagopalan *et al.*, 2003). More recent attempts at therapeutic angiogenesis have also been negative. Poole *et al*, randomised 159 patients to receive granulocyte-macrophage colony-stimulating factor (GM-CSF), or placebo, injections over four weeks(Poole *et al.*, 2013). Despite the hope that GM-CSF would mobilise bone marrow derived endothelial progenitor cells to stimulate vascular repair, improve endothelial function, and angiogenesis, there was no improvement in walking performance, ABPI, or quality of life scores at 3 months. In a somewhat similar way, Perin *et al*. randomised n=82 patients with claudication to received harvested bone marrow-derived aldehyde-dehydrogenase bright cells, or placebo, injected into the thigh and calf muscles (Perin *et al.*, 2017). At six months, there was no significant difference in treadmill walking ability, nor anatomic or physiologic imaging endpoints, such as ABPI and collateral growth. In another randomised clinical trial of n=210 patients with claudication, McDermott *et al*. assessed the potential adjuvant effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in addition to supervised exercise, finding no effect on six-minute walk distance at 3 months follow-up (McDermott *et al.*, 2017a).

Extracorporeal shockwave therapy (ESWT) is a non-invasive therapy that is hypothesised to induce angiogenesis through mechano-transduction high frequency sound. In an early feasibility study, Harwood *et al*. randomised 30 patients with claudication to nine 20-minute sessions of shockwave therapy over three weeks, or to sham condition (Harwood *et al.*, 2017). At 12-weeks follow-up, patients receiving real shock therapy improved their treadmill pain-free and maximum walking distance by 276% and 167%, respectively ($p<0.05$).

Transcutaneous electrical nerve stimulation (TENS) was evaluated in a small pilot study finding that 45-minutes of stimulation at 80 or 100 Hz seemed to improve treadmill walking performance compared to sham or control condition (Labrunée *et al.*, 2015). The TENS-PAD study is a multicentre randomised placebo (sham) controlled trial, that is presently recruiting, aiming to evaluate the effect of adjuvant TENS therapy to supervised exercise therapy (Besnier *et al.*, 2017).

### 2.4.5 **Economic Impact**

Total health expenditure in Australia has been steadily growing over the last few decades (Australian Institute of Health and Welfare, 2016). The most recent government data from the 2016-16 Health expenditure Australia report show that total health spending has increased to AU$170.4 billion (Australian Institute of Health and Welfare, 2017c). For the first-time health expenditure now represents over 10% of gross domestic product. A report from 2014 estimates expenditure for cardiovascular disease to be approximately AU$7.74 billion (Australian Institute of Health and Welfare, 2014a). There is very little contemporary data with regard to the expenditure for PAD in Australia due to limitations in data collection (Australian Institute of Health and Welfare, 2014b). The most recent government report from over 20 years ago, estimated the total cost attributable to PAD to be AU$179 million (Waters, Armstrong & Senes-Ferrari, 1998), but this is clearly outdated. A present-day estimate of direct expenditure can be calculated by multiplying an estimate of the prevalence of PAD with the annual cost of healthcare per patient (Delaney, 2015). In the analysis from the Australian REACH registry, Ademi *et al.* calculated the median annual total cost for a patient with PAD to be $AU12,651 (Ademi *et al.*, 2010). Assuming a prevalence of PAD

of 4.3% (Selvin & Erlinger, 2004) among the 11.41 million Australians >40 years old (Australian Bureau of Statistics, 2018b), the total annual cost is estimated to be AU$6.21 billion. For comparison, the total health care system expenditure for cancer was AU$ 4.53 billion in 2008-09 (Australian Institute of Health and Welfare, 2013). This extrapolation is of course limited by a number of assumptions, such as differing age distribution and ethnic backgrounds in two studies used to calculate the estimate. In addition, this calculation does not capture the indirect costs to the economy which include, but are not limited to, reduced employment, lost earnings and absenteeism, carers costs, allied health costs and mortality burden. Nevertheless, it is clear that PAD represents one of the most significant cost burdens on the Australian economy through both direct and indirect costs.

# 3 METHODOLOGY

Methodology common to the overall study is presented here. Methodology specific to individual studies is presented in their respective Chapters.

## 3.1 **Study Design and Setting**

This study conducted as a non-randomised prospective cohort study of patients with intermittent claudication undergoing supervised exercise therapy.

The study was conducted in the Southern Adelaide region at the Flinders Medical Centre, which is the sole publicly funded vascular surgical referral centre for the Southern Adelaide Local Health Network (SALHN). The Department of Vascular Surgery at Flinders Medical centre services a population of approximately 440,000 people residing in area of approximately 25,576km$^2$ (Australian Bureau of Statistics, 2018a). Patients were recruited from a specific vascular surgery outpatient clinic, known as the Claudication Clinic, specialising in the assessment and management of patients with intermittent claudication. This clinic receives referrals from primary care physicians and other specialists including vascular surgeons. Patients attending the Claudication Clinic were initiated on appropriate medical therapy such as antiplatelet, statin and antihypertensive therapy if appropriate. In addition, patients received counselling with regard to smoking cessation and other lifestyle modifications to improve cardiovascular risk (regular exercise, healthy diet, and weight loss). When needed, patients were referred to allied health services such as dietitians, diabetic educators and the nurse-led tobacco cessation service. All suitable patients were then offered a 12-week program of twice-weekly supervised treadmill walking, which took place at nearby rehabilitation hospital, The Repatriation General Hospital. Running

parallel to the first six weeks of the program, a series of six educational sessions was provided to patients. The educational sessions covered the cause and natural history of the disease, lifestyle modification and pharmacological therapies, the benefits of exercise therapy, the important of healthy feet, and invasive management and outcomes. The timetable of the exercise program and education sessions is shown in Appendix A.

## 3.2 **Study Population and Eligibility**

Patients who agreed to participate in the supervised exercise program between January 2013 and December 2014 were screened for eligibility into the study. Inclusion criteria consisted of a clinical history of calf claudication along with low ankle brachial pressure index (ABPI) <0.9 and radiographic evidence of infra-inguinal disease in the absence of significant aorto-iliac disease. This allowed for an anatomically homogenous patient cohort. Patients were excluded if they (1) had evidence of critical limb ischaemia (rest pain or tissue loss), (2) had recently (<12 months) undergone peripheral vascular revascularization, (3) had recent exercise training or cardiac rehabilitation, (4) suffered blood dyscrasias or were anticoagulated or, (5) suffered from cardio-respiratory or arthritic morbidities which limited exercise capacity.

Additional data on patients who were part of a treadmill-only training arm of a randomised controlled trial conducted at the same institution were also included in the data analysis (Delaney *et al.*, 2014). Inclusion and exclusion criteria and exercise training protocol were similar to the present study and are described further below.

## 3.3 **Procedure for Informed Consent**

The study was approved by the Southern Adelaide Human Research Ethics

Committee (Application number: 139.10).

Patients offered enrolment into the study after receiving a verbal description of the nature of the study and procedures. In addition, patients were provided with a written patient information sheet and consent form. All included patient's provided written informed consent. Patients who refused to participate in the study were not prevented from attending all supervised exercise sessions and education sessions.

## 3.4 **Exercise Intervention**

The SEP ran over three months 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 a senior clinical physiotherapist or exercise physiologist with experience in administering exercise interventions for patients with cardiorespiratory disease. In accordance with international guidelines at the time of the study, participants were instructed to begin walking at a speed and incline to induce claudication pain within 3–5 minutes, stop and rest until pain abated, then repeat for the duration of the 1 hour session (Norgren *et al.*, 2007). Initial treadmill speed was commenced at the average speed calculated from the result of the individual's initial 6-minute walk distance (6MWD) test. During each training session, the physiotherapist monitored the patient's progress to ensure onset of symptoms ≤5 minutes of walking. If patients were able to walk >5 minutes without symptoms, the workload of the treadmill was first adjusted by increasing the speed up to the patient's maximum safe walking speed and then by increasing the incline.

## 3.5 Data Collection

Participants in the study were assessed within one week prior to commencing the supervised exercise program, and again within one week following its completion.

### 3.5.1 Routine Assessment

Once patients were assessed, diagnosed and enrolled into the study, they attended a research clinic at a separate appointment further data collection.

#### 3.5.1.1 Clinical Assessment

Participants had their medical, surgical and medication histories and examination findings documented.

#### 3.5.1.2 Ankle Brachial Pressure Index Measurement

All patients underwent ABPI measurement. Participants were rested in the supine position for 20-30 minutes, after which systolic blood pressure was measured in both arms using an appropriately sized blood pressure cuff (Pickering *et al.*, 2004) and handheld 8 MHz high-sensitivity wide beam continuous wave Doppler device (Huntleigh Diagnostics Ltd., Cardiff, United Kingdom). Ankle pressure was measured in a similar way, and the ABPI was calculated as the highest ankle pressure from each limb divided by the highest of the brachial pressures (Jones *et al.*, 2017; Aboyans *et al.*, 2018).

#### 3.5.1.3 Biochemical Assessment

Participants were instructed to fast for 8-12 hours overnight and to withhold all medications. Participants underwent routine phlebotomy and blood collection for traditional cardiovascular risk factors and inflammatory markers. Full blood panel, leukocyte and monocyte counts were measured with automated cell counters using standard techniques. Lipid levels (total-, high-density lipoprotein

(HDL), low-density lipoprotein (LDL), triglycerides, blood glucose, HbA1c, C-reactive protein, creatinine, and homocysteine were measured immediately under routine hospital conditions.

### 3.5.2 **Retention and Compliance**

Satisfactory compliance with the SEP was defined if participants attended and completed at least 80% of the scheduled exercise sessions. Regular phone contact was made with the participants to ensure their well-being and encourage their continued involvement in the study. Participants who had difficulty into attending the scheduled exercise sessions due to transport issues were provided with taxi vouchers for the duration of the program.

Participants were considered 'responders' if they demonstrated a ≥30% increase in pain-free walking-distance (PFWD) at the completion of 12-weeks of SEP training

### 3.5.3 **Assessment of Outcome Measures**

#### 3.5.3.1 **Exercise Testing and Walking Performance**

A separate appointment was completed for assessment of walking performance. All participants were assessed by a physiotherapist within the week prior to commencing the exercise program, and within one week following completion of the program using a standardised over ground six-minute-walk test (Nordanstig *et al.*, 2014a; Hirsch *et al.*, 2006; McDermott *et al.*, 2014). Distance at first sign of claudication (PFWD) and total distance covered in six minutes were recorded (6MWD).

#### 3.5.3.2 **Endothelial Function**

Endothelial function measurements are used in Chapters 5, 6, and 7.

Patients underwent sequential endothelial function testing after a standardised 15-minute rest period.

##### 3.5.3.2.1 **Brachial Artery Flow Mediated Dilation**

The FMD technique was based on recently published consensus guidelines (Thijssen *et al.*, 2011). The participants 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 (Figure 3.1). A stereotactic probe holder, together with a high-resolution linear 6-13 MHz probe (SonoSite HLF38x) and an ultrasound system (SonoSite M-Turbo, SonoSite, Inc, Bothel, USA) with 3-lead ECG recording was used to obtain long-axis images of the brachial artery. Images were obtained at baseline, and during forearm hyperaemia for 240 seconds following 5 minutes of forearm cuff occlusion. The resultant DICOM images were then analysed using the Brachial Artery Analyser software package (MIA-LLC, Coralville, USA). ECG recording allowed the gated measurement of brachial artery diameter during the systolic peak (Figure 3.3). The FMD was calculated by obtaining the percentage increase in diameter of the brachial artery following hyperaemia (Figure 3.2). The maximum percentage increase was calculated from pre-occlusion diameter and the maximum post occlusion diameter as per standard FMD technique (Thijssen *et al.*, 2011).

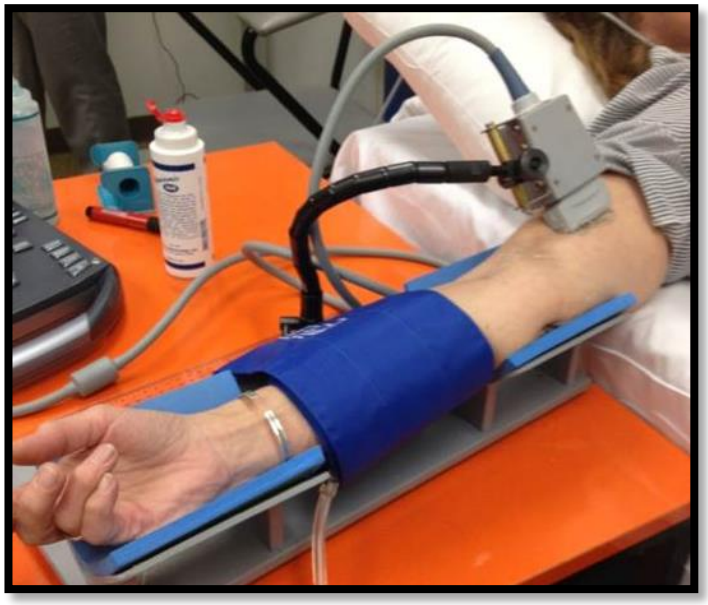

Figure 3.1. Technique for FMD. The patient is supine with the arm abducted on a level table supported in a specially designed cradle. The ultrasound probe is positioned then fixed into position for the duration of the test. Image courtesy of Mr Richard Allan, Flinders Medical Centre.

$$\text{FMD (\%)} = \left[\frac{(\text{max diameter} - \text{baseline diameter})}{\text{baseline diameter}}\right] \times 100$$

Figure 3.2. Equation used to calculate brachial FMD.

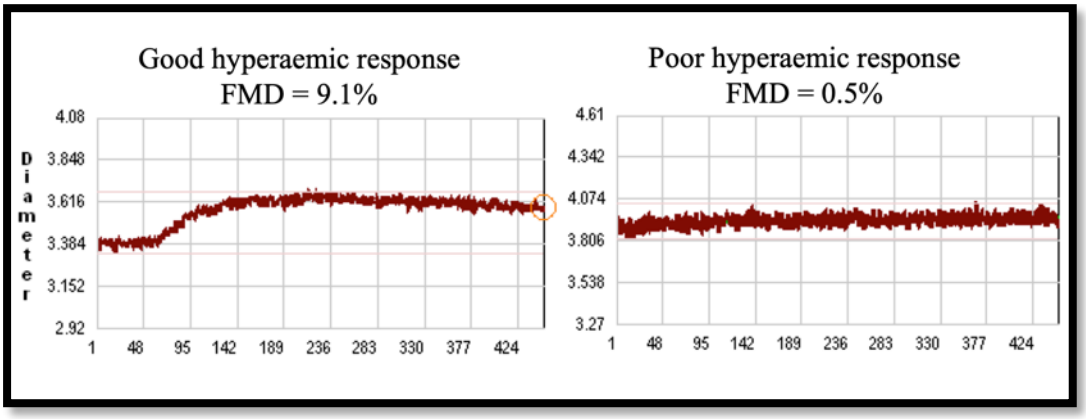


Figure 3.3. Brachial artery diameter measurements performed by the Brachial Artery Analyzer software during the hyperaemic period. On the left there has been an increase in brachial artery diameter indicating preserved endothelial function and a FMD of 9.1%. On the right there has been little dilation of the brachial artery indicating a poor hyperaemic response, and low FMD of 0.5%. Image courtesy of Mr Richard Allan, Flinders Medical Centre.

#### 3.5.3.2.2 **Peripheral Artery Tonotomy**

The reactive hyperaemia index (RHI) was obtained using an EndoPAT peripheral arterial tonometry device (Itamar Medical Ltd, Caesarea, Israel) according to the manufacturer's instructions. To avoid known confounders, patients were fasted overnight for 8-12 hours, and had vasoactive drugs withheld. In the siting position pneumatic pressure sensitive finger probes were connected to the participants index finders bilaterally (Figure 3.4). Via these probes the pulse waveform was continuously recorded. After a 5min baseline period, a pneumatic cuff was inflated on the right upper arm to occlude the brachial artery for 5 minutes, followed by 5 minutes after cuff release. Reactive hyperaemia index was measured using the proprietary software on the EndoPAT device (Figure 3.5).

Figure 3.4. EndoPAT setup and positioning showing the forearms resting in specifically designed cradles. The inset shows the position of the modified plethysmographic bio-sensors on the tip of the index finger. Image courtesy of Mr Richard Allan.

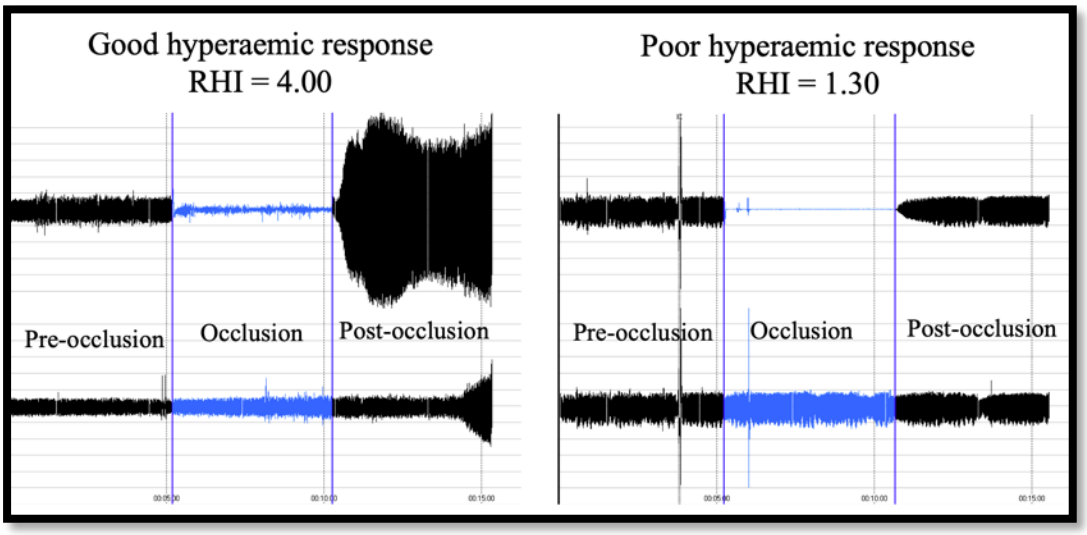


Figure 3.5. EndoPAT responses provided by the manufacturers software. The upper tracing shows brachial artery occlusion and the post occlusion response, on the left a significant increase in pulse wave amplitude. On the right there is a poor increase in pulse wave amplitude. The lower tracings record the pulse wave amplitude on side without brachial artery occlusion. Image courtesy of Mr Richard Allan, Flinders Medical Centre.

## 3.6 **Statistical Methods**

GraphPad Prism 6.00 for OS X (GraphPad Software, La Jolla, CA, USA) was used for all statistical calculations. All tests were performed 2-sided and a significance level of $p<0.05$ was considered to indicate statistical significance. See individual chapters for statistical methods employed in each study.

# 4 THE EFFECT OF TREADMILL EXERCISE ON REGIONAL LOWER LIMB SKELETAL MUSCLE MASS

## 4.1 **Abstract**

*Objectives*

Supervised exercise is currently recommended for the first line treatment of intermittent claudication based on improvement in walking capacity. However, concerns regarding the promotion of skeletal muscle atrophy by repetitive ischaemia-reperfusion due to treadmill-based programs remain. Since preservation of skeletal muscle mass (SMM) is integral to functional capacity and longevity, this study measured the effect of standard treadmill-based supervised exercise on regional lower limb skeletal muscle mass in patients with intermittent claudication. The primary hypothesis was that SMM would decrease in the symptomatic calf muscles of patients with intermittent claudication.

*Methods*

Patients with calf claudication due to infra-inguinal peripheral artery disease underwent whole body dual energy x-ray absorptiometry (DEXA) before and after completion of a 3-month supervised treadmill exercise program. Lower limb SMM was analysed depending on the region of the lower limb (thigh vs. leg) and side of symptoms. Walking performance was assessed using pain free walking distance (PFWD) and six-minute walking distance (6MWD) tests.

*Results*

Thirty-six patients with calf claudication completed exercise training and DEXA scanning, allowing analysis of 55 symptomatic and 17 asymptomatic lower limbs. No difference in SMM in either symptomatic ($p=0.53$) or asymptomatic lower legs ($p=0.59$) was detected following the program. In contrast, a significant

decrease in SMM was observed in both symptomatic (p=0.04) and asymptomatic thighs (p=0.005) following exercise training. Both PFWD (p=0.001) and 6MWD improved significantly (p=0.004) but were not associated with changes in SMM.

*Conclusions*

Three months of standard treadmill-training for intermittent calf claudication did not result in loss of leg SMM, however a significant decrease in bilateral thigh muscle mass was observed even in patients with unilateral symptoms. Further work is required to determine pathophysiology and impacts of these changes on function and survival.

## 4.2 **Introduction**

Ischaemic muscle pain affecting the legs is the most common manifestation and most common reason for patients with PAD to seek medical treatment. All modern clinical guidelines recommend supervised walking exercise as the first line of treatment based on growing evidence of short-term improvements in walking performance (Norgren *et al.*, 2007; Askew *et al.*, 2014; Conte *et al.*, 2015; Lane *et al.*, 2017; Gerhard-Herman *et al.*, 2017; Aboyans *et al.*, 2018). In contrast to interventions for other cardiovascular diseases, there is very little evidence on whether supervised exercise can improve mortality. Only one retrospective study has reported on the effect of participating in a supervised exercise program (Sakamoto *et al.*, 2009). In addition, little is known about the exact mechanisms which lead to improved walking performance. Alterations to muscle function are thought to explain some of the improvement in walking performance as a result of supervised exercise programs (SEPs) with a number of studies demonstrating changes in muscle architecture, mitochondrial content and fibre type (Beckitt *et al.*, 2012; Brizendine *et al.*, 2014). These adaptations are currently thought to be beneficial, however, repetitive exercise of an ischaemic muscle group is accompanied by an ischaemia-reperfusion phenomenon, which may be deleterious to muscle function, and perhaps the whole patient, longer term. Indeed, people with PAD have significantly smaller calf muscle area compared to controls without PAD (McDermott *et al.*, 2007b). Furthermore, in patients with unilateral PAD, calf muscle area is significantly reduced in the leg with the lowest ABPI compared to the leg with the highest ABPI, and lower ABPI correlates with lower calf muscle area, implicating muscle ischaemia as a direct negative factor on skeletal muscle mass (McDermott *et al.*, 2007b).

In addition to muscle area, which is a surrogate for muscle mass, lower limb muscle strength is adversely affected by chronic ischaemia, with lower ABPI values associated with weaker lower limb strength in multiple functional tests (McDermott *et al.*, 2008b, 2004a). Thus, it is conceivable that since regular exercise provokes episodic ischaemia and reperfusion, it may adversely affect calf muscle structure and function. Indeed, treadmill-based exercise programs have not been found to improve lower limb strength (Hiatt *et al.*, 1994; McDermott *et al.*, 2009a). On the contrary, a clinical trial demonstrated a loss of bilateral leg strength when patients change from a resistance-training program to a treadmill-training program (Hiatt *et al.*, 1994). Given the known association between lower limb strength and mortality in patients with PAD, preservation of strength and muscle mass is unquestionably important (Morris *et al.*, 2014). However, a recent randomized controlled trial that compared standard treadmill-walking training to combination walking and resistance exercise, demonstrated that the treadmill-only exercise program elevated the activity of muscle calpain, a protease implicated in myocyte damage and apoptosis, whereas combination training did not (Delaney *et al.*, 2014). In addition, this patients who completed 12-weeks of treadmill-only training showed a decline lean muscle mass compared to those who completed the combined training (Delaney *et al.*, 2014). Together this suggests that prolonged exercise of muscle groups under ischaemic conditions may accelerate muscle atrophy. It is not known, however, if the changes in muscle mass are regional, affecting proximal or distal muscle groups related to blood supply. The purpose of this study was therefore to test the hypothesis that standard treadmill-training for calf claudication adversely affects calf skeletal muscle mass (SMM) despite improvements in walking performance, in patients with calf claudication secondary to infra-inguinal peripheral artery disease. The primary hypothesis was

that SMM would decrease in the symptomatic calf muscles of patients with intermittent claudication.

## 4.3 **Methods**

Refer to Chapter 3 for Common Methods: 3.1 Study Design and Setting, 3.2 Study Population and Eligibility, 3.3 Procedure for Informed Consent, 3.4 Exercise Intervention, 3.5.1 Routine Assessment, 3.5.3.1 Assessment of Exercise and Walking Performance.

### 4.3.1 **Regional Skeletal Muscle Mass Quantification**

Whole body composition scanning was performed using a DEXA system (Lunar Prodigy, GE Healthcare, UK) with GE EnCORE software (GE Healthcare, v. 10.51.006). Participants were positioned supine on the DEXA table top with their feet in a neutral position and hands flat by their sides. After acquisition, whole-body composition was estimated using default automated segmentation. Appendicular soft tissue lean mass was then used to determine total body skeletal muscle mass according to previously validated methods (Kim *et al.*, 2002).

For regional body composition, custom regions of interest (ROI) were traced manually over the DEXA planogram to segment the lower limb into thigh and leg regions. This study defined the thigh as the part of the lower limb between the hip and knee joint and the calf as the part of the lower limb below the knee joint. ROI borders were adapted from previously validated methods (Burkhart, Arthurs & Andrews, 2009). The proximal extent of the thigh ROI was modified because it was defined by an angled line connecting the lateral aspect of the anterior superior

iliac spine and the inferior ramus of the pubis. This line is influenced by sex, with men having a more vertical line than women, given their taller and narrower pelvic structure. The potential effect of this is exclusion of proximal medial thigh musculature and contamination by lower abdominal mass, particularly in obese individuals. Thus, the thigh ROI used in this study was defined proximally by an angled line drawn from the centre of the pubic symphysis to the superior aspect of the greater trochanter, distally by a horizontal line through the centre of the knee joint space, and medially and laterally to include all soft tissue. The calf ROI was defined proximally by a horizontal line through the centre of the knee joint space and distally to include the foot and toes; medially and laterally, all soft tissue was included. **Error! Reference source not found.** demonstrates the DEXA segmentation for a representative patient.

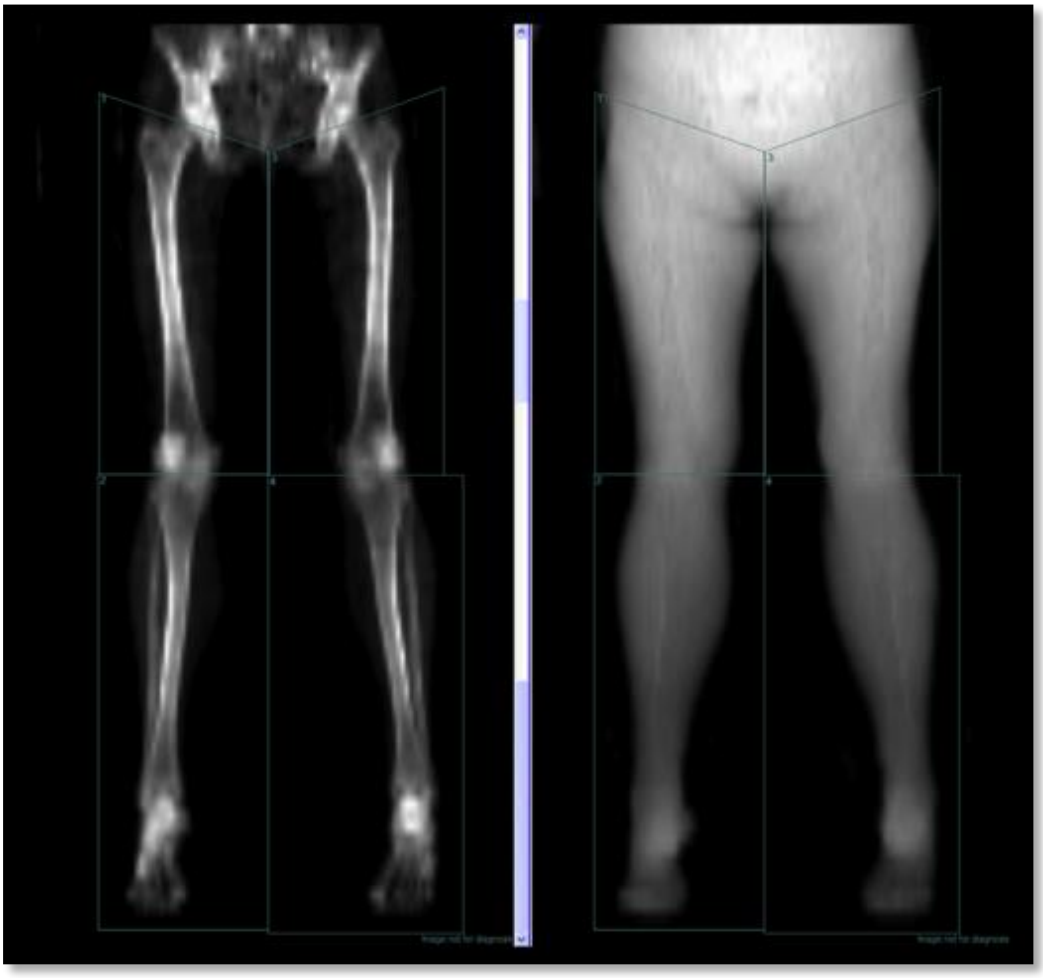

Figure 4.1. Dual-energy X-ray absorptiometry planograms windowed for skeleton and soft tissue. Boxes are drawn around the regions of interest using the definitions described above.

### 4.3.2 **Validation of Manual Segmentation**

To validate this method of manual segmentation, two assessors used the above definitions to independently segment DEXA scans from 15 randomly selected patients. One assessor repeated the measurements >1 week later. Measurements of skeletal muscle mass were recorded, and the Pearson correlation coefficient (r), mean coefficient of variation, and intraclass correlation coefficients (ICCs) were used to quantify between and within-assessor reliability (Shrout & Fleiss, 1979).

Good to excellent reliability was accepted if ICC was ≥0.75 (Portney & Watkins, 2000)

### 4.3.3 **Statistical Analysis**

Categorical data is expressed as n (%) and differences between groups for categorical data were tested using the $\chi^2$ test. Summary continuous variables are expressed as arithmetic means and standard deviation (SD). The Student paired t-test was used for within-group comparisons and independent samples t-test was used for between group comparisons. Because symptoms were unilateral in some patients and bilateral in others, data from each leg were considered as separate entities. The Pearson correlation was used to assess the association among changes in PFWD, 6MWD, skeletal muscle mass across limb region and symptom status. For correlations, the limb with the lowest ABPI was used when symptoms were bilateral.

#### 4.3.3.1 **Sample Size Justification**

For the primary outcome of limb SMM, sample size was calculated based on the assumption of an effect size (Cohen's d) of 0.5 (mean of differences 75g of SMM, SD of the difference 150g). Using G*Power software version 3.1 for OSX (Düsseldorf, Germany), the sample size required for a two-sided statistical test of the difference between two dependent means of matched pairs was calculated *a priori*. Assuming an effect size of 0.5, $\alpha = 0.05$, and $\beta = 0.80$, a total sample size of 34 pairs would be required.

## Results

Between February 2013 and December 2014, 98 patients were assessed for eligibility into the study. Excluded were 16 patients with no evidence of PAD causing their symptoms, 28 with suitable disease who refused to undergo exercise therapy, 24 who met exclusion criteria (5 unable to walk on treadmill, 4 with poor cardiorespiratory status, 4 critical limb ischaemia, 9 aortoiliac disease, 1 warfarin therapy, and 1 large abdominal aortic aneurysm requiring surgery), 4 who participated in exercise therapy but did not consent to the study, and 1 patient who was later excluded after attending <80% of treadmill sessions. Data from the remaining 25 participants was combined with data from 11 participants from the treadmill-training arm who completed >80% of the training program and also underwent DEXA scans before and after training (Delaney *et al.*, 2014). Baseline demographic, laboratory data, and medications of the combined group are reported in Table 3.1 and Table 3.2 respectively.

The modified ROI measurements described were highly reproducible within- and between-raters as shown in Table 3.3. All measurements demonstrated a Pearson correlation coefficient >0.99, a mean coefficient of variation <2%, and ICC ranging from 0.997 to 1.000 ($p < 0.0001$).

The main study outcomes are reported in Table 3.4.

Walking outcomes, PFWD and 6MWD, significantly improved following the 12-week supervised exercise program (mean, 47 (SD, 76) m; p=0.001) and (mean, 26 (SD, 50) m; p=0.004). Following the SEP, there was no significant change in whole body skeletal muscle mass (mean change -0.10 (SD, 0.72) kg: p=0.41), nor the lean mass of symptomatic calves (mean change 9 (SD, 100) g; p=0.53) or asymptomatic calves (mean change 35 (SD, 260) g; p=0.59). In contrast, there

was a significant reduction in lean mass of thighs on both symptomatic (mean -61 (SD, 220), p=0.043) and asymptomatic sides (mean -94 (SD 120) g; p=.005). No significant correlation was observed between changes in lean mass and changes in walking performance measures. Symptomatic-side thighs (ΔLM vs ΔPFWD r=-0.09, p=.62), (ΔSMM vs Δ6MWD r=-0.02, p=0.91), asymptomatic-side thighs (ΔSMM vs ΔPFWD r=-0.25, p=0.47), (ΔSMM vs Δ6MWD r=-0.5, p=0.09), symptomatic calves (ΔSMM vs ΔPFWD r=-0.03, p=0.87), (ΔLM vs Δ6MWD r=-0.32, p=0.058), asymptomatic calves (ΔSMM vs ΔPFWD r=0.52, p=0.1), (ΔSMM vs Δ6MWD r=0.31, p=0.3)

Table 4.1. Participant characteristics at baseline in those undergoing assessment of skeletal muscle mass by DEXA.

| **Variable** | **No.** | **Result** | |
|---|---|---|---|
| Age, mean (SD), years | 36 | 72 | (10) |
| Sex | | | |
| Male (%) | | 26 | (72) |
| Female (%) | | 10 | (28) |
| BMI, mean (SD), kg/m$^2$ | 36 | 27.6 | (4.1) |
| Current smokers, No. (%) | 25 | 10 | (43) |
| Former smokers, No. (%) | 25 | 12 | (52) |
| Pack-years, mean (SD) | 25 | 40.3 | (24) |
| Diabetics, No. (%) | 27 | 7 | (26) |
| Hypertension, No. (%) | 27 | 22 | (81) |
| Dyslipidaemia, No. (%) | 27 | 23 | (85) |
| Ischaemic heart disease, No. (%) | 27 | 8 | (30) |
| AAA, No. (%) | 26 | 4 | (15) |
| COPD, No. (%) | 25 | 2 | (8) |
| ABPI, mean (SD) | | | |
| Lowest resting | 36 | 0.71 | (0.21) |
| Lowest post-exercise | 34 | 0.45 | (0.33) |
| Asymptomatic leg | 17 | 0.98 | (0.17) |
| Medications | | | |
| ACE-I/ARA, No. (%) | 27 | 22 | (81) |
| Beta-blockers, No. (%) | 26 | 8 | (30) |
| Aspirin, No. (%) | 26 | 21 | (80) |
| Clopidogrel, No. (%) | 26 | 4 | (15) |
| Statins, No. (%) | 27 | 21 | (78) |

AAA, Abdominal aortic aneurysm; ABPI, ankle-brachial index; ACE-I, angiotensin-converting enzyme inhibitor; ARA, angiotensin receptor antagonist; BMI, body mass index; COPD, chronic obstructive pulmonary disease; SD, standard deviation.

Table 4.2. Laboratory values at baseline in those undergoing assessment of skeletal muscle mass by DEXA.

| **Variable** | **No.** | **Mean** | **SD** | **Ref Range** |
|---|---|---|---|---|
| Haemoglobin, g/L | 34 | 143 | (16) | ♂: 130-170<br>♀: 120-150 |
| White cells, $x10^9$/L | 34 | 7.3 | (2.3) | 4-10 |
| Neutrophils, $x10^9$/L | 34 | 4.8 | (1.8) | 2-7 |
| C-reactive protein, mg/L | 33 | 6.4 | (24) | <5 |
| Creatinine, µmol/L | 33 | 79 | (19) | ♂: 60-110<br>♀: 45-90 |
| Albumin, g/L | 31 | 41 | (8) | 33-48 |
| Total cholesterol, mmol/L | 34 | 4.0 | (1.1) | <4.0 |
| HDL, mmol/L | 34 | 1.4 | (0.5) | 1.0-2.2 |
| LDL, mmol/L | 34 | 2.1 | (0.9) | 2.0-3.4 |
| Triglycerides, mmol/L | 34 | 1.4 | (0.7) | <2.0 |
| Homocysteine, µmol/L | 31 | 12.7 | (3.8) | 5-15 |
| $HbA_1c$ (%) | 26 | 6.1 | (0.9) | 3.5-6 |

HbA1c, Glycosylated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

Table 4.3. Reliability testing of custom region of interest (ROI) segmentation.

| Region of interest | Pearson's r | Mean CoV (%) | ICC (95% C.I.) |
|---|---|---|---|
| Intra-observer | | | |
| Leg | 0.9993 | 0.3993 | 1.00 (0.999 – 1.000) |
| Thigh | 0.9970 | 0.8993 | 0.998 (0.996-0.999) |
| Inter-observer | | | |
| Leg | 0.9978 | 0.544 | 0.999 (0.998-0.999) |
| Thigh | 0.9961 | 1.231 | 0.997 (0.989-0.999) |

CI, Confidence interval; CoV, coefficient of variation; ICC, intraclass correlation coefficient. $p < 0.0001$ for all tests.

Table 4.4. Skeletal muscle mass study outcomes.

| Variable | No. | Baseline, mean (SD) | | 12-week, mean (SD) | | Change, mean (SD) | | p-value |
|---|---|---|---|---|---|---|---|---|
| PFWD, m | 33 | 164.7 | (77.68) | 213.3 | (93.18) | 47.06 | (75.6) | 0.001 |
| 6MWD, m | 36 | 395.1 | (78.02) | 420.8 | (68.04) | 25.64 | (50.3) | 0.004 |
| Total body SMM, kg | 36 | 24.16 | (6.18) | 24.06 | (6.15) | -0.1 | (0.72) | 0.41 |
| Calf muscle mass | | | | | | | | |
| Symptomatic side, g | 55 | 2671 | (607.6) | 2680 | (594.0) | 8.53 | (101) | 0.53 |
| Asymptomatic side, g | 17 | 2701 | (567.3) | 2736 | (559.4) | 35.35 | (257.2) | 0.59 |
| Thigh muscle mass | | | | | | | | |
| Symptomatic side, g | 55 | 5826 | (1502) | 5764 | (1460) | -61.18 | (218.4) | 0.043 |
| Asymptomatic side, g | 17 | 5684 | (1369) | 5590 | (1366) | -94.35 | (119.9) | 0.005 |

6MWD, 6-minute walk distance; PFWD, pain-free walking distance; SD, standard deviation; SMM, skeletal muscle mass.

## 4.4 **Discussion**

The improvements in walking performance following supervised treadmill testing are well documented (Fokkenrood *et al.*, 2013) and were also observed in the present study of a typical claudicant population. In contrast, the impact of treadmill based supervised exercise training on regional lower limb lean mass has not previously been investigated. Importantly our data has shown that completion of such training is not accompanied by a loss of lean mass in the symptomatic calves of patients, generally considered the ischaemic end-organ in patients with infra-inguinal arterial disease (Lindbom, 1950; Mavor, 1956).

One of the key findings of this study is that no change in SMM was observed in the asymptomatic calves of claudicant participants. This casts doubt on the hypothesis that the regional ischaemia-reperfusion experienced by exercising claudicant individuals promotes skeletal muscle atrophy. However, muscle mass alone is probably not the only determinate of strength and function (Barbat-Artigas *et al.*, 2013). In support of this, changes in muscle fibre composition have been observed following supervised exercise. Ambrosio *et al*. used protein electrophoresis studies to show reduced proportions of type I to type II fibres in the muscles of patients with claudication versus controls, which is reversed by exercise training (Ambrosio *et al.*, 2000). Beckitt *et al*. observed that 3 months of supervised treadmill training resulted in a proportional increase in myosin heavy chain type I expression in symptomatic calf muscles and that this correlated with improved walking performance (Beckitt *et al.*, 2012).

Surprisingly, however, a small but statistically significant reduction in SMM was observed in the thighs from both asymptomatic and symptomatic sides after the exercise program was completed. The cause and importance of this observation is

currently unknown but is consistent with a previous randomized trial of exercise programs where patients lost strength in both lower limbs after crossing over to treadmill training from strength training (Hiatt *et al.*, 1994). Furthermore, it may explain why treadmill-only training programs fail to improve performance-based tests of function such as chair stand, gait speed and standing balance, which are rely proximal limb strength (McDermott *et al.*, 2009a).

In further support of a link between distal ischaemia and proximal muscle groups is the observation that the strength of flexors and extensors, of hip and knee, are highly correlated with the severity of the peripheral ischaemia, defined by ABPI (McDermott *et al.*, 2004a). Moreover, biomechanical gait analysis of patients with intermittent claudication has demonstrated reduced hip, knee, and ankle power on commencement of walking, which worsens after the onset of pain but is also apparent on the unaffected side in unilateral disease (Koutakis *et al.*, 2010b, 2010a). Together, this suggests that despite symptoms predominantly localising to calf muscles on the symptomatic side, there are adverse effects observed in more proximal muscles and contralateral limb muscles remote to areas of reduced arterial perfusion. Possible mechanistic explanations for this affect may lie in systemic humoral pathways, such as oxidative stress, alternatively it may involve neuropathy. In support of this, Koopman *et al*. performed electrophysiological studies on patients with unilateral claudication and found evidence of neuropathy in both symptomatic and asymptomatic limbs (Koopman, de Vries & de Weerd, 1996). In addition, histological, and electrophysiological examinations of muscle and nerves from both ischaemic, and non-ischaemic limbs have revealed evidence of both sensory and motor nerve dysfunction (England *et al.*, 1992; Weber & Ziegler, 2002).

Whether exercise therapy can reverse these neuromyopathic gait, and functional abnormalities, which have received little research attention to date, is currently unknown. England *et al*. reported that mixed walking and strength training preserved calf muscle strength but did not worsen peripheral neuropathy (England *et al.*, 1995). Conversely, Hiatt *et al*. demonstrated that although treadmill training increased peak exercise performance, it was associated with increased skeletal muscle denervation histologically (Hiatt *et al.*, 1996). Whether dysfunction or denervation of nociceptor afferents also occurs is unknown but is an important consideration because the main justification of walking exercises is suppression of walking-induced pain. One could speculate the nociceptor denervation could increase PFWD. Nevertheless, it could be suggested that exercise therapy alone might not be sufficient to reverse the neuropathic changes observed in PAD and that treadmill training alone may be detrimental to other ambulatory function that relies on proximal muscle mass, despite improvements in walking tests. Alternatively it could be hypothesised that loss of thigh muscle mass is simple an adaptation to the volume and nature of treadmill walking, which is known to result in more proximal muscle activation than over ground walking. Even though thigh activation is greater in treadmill walking, it is not a hypertrophic stimulus in the way that resistance training is. Thus the loss of proximal muscle mass in treadmill trained patients with claudication may be analogous to athletic adaptations see in competitive endurance athletes who lack muscularity in favour of cardiorespiratory efficiency. Combining these considerations, the findings of the present study challenge the conclusion that enough is known about the effects of supervised exercise training to settle the debate about the optimal treatment of claudication.

The limitations of this study are acknowledged. Firstly, the aim of this study was to focus on patients with claudication undergoing treadmill-only training as this is the most often recommended form of exercise. Without a control group, certainty that the changes observed would not also be seen in healthy controls, cannot be achieved. In addition, the observed results cannot be extrapolated to programs that have a strength-training element. However, it is likely that programs with a strength-training element will lead to increased muscle mass as previous demonstrated (Delaney *et al.*, 2014).

Secondly, daily physical activity of patients was not monitored outside of training; thus, we cannot be assured it did not decrease, despite all patients being instructed to maintain their normal activity levels. In addition, there was slight variation in the instructions given to patients regarding at what level of pain to cease exercise. This may have resulted in a more profound ischaemia-reperfusion injury in some of the group and is indeed an area that requires more study.

Thirdly, the measurement of SMM is an anatomic assessment that sheds little light on muscle performance and quality. It is possible that despite SMM reductions, muscle performance improved through changes at the fibre level. In addition, the follow-up duration was short, and it is not possible to speculate whether the changes in muscle mass would eventually be observed in the calves or whether there were favourable or deleterious consequences.

Future studies should avoid focusing only on symptomatic muscles but should include assessment of the contralateral and proximal muscle groups, which are also affected by PAD. Also, a shift away from end points dependent on pain perception and broadening of out- comes to include strength and functional measures may help identify the optimal exercise prescription (Parmenter,

Raymond & Fiatarone Singh, 2013). Finally, the paucity of data on long-term outcomes after exercise therapy needs to be addressed.

## Conclusion

In this study investigating regional lower limb SMM in patients undergoing 12 weeks of standard treadmill training for intermittent claudication, no loss of calf lean muscle mass was observed, casting doubt on the hypothesis that repetitive ischaemia-reperfusion prevents maintenance of calf SMM. In contrast, a significant decrease in bilateral thigh SMM was observed even in patients with unilateral symptoms. Further work is required to determine whether these changes affect strength, functional performance, quality of life, and survival, considering the exercise modality and the limitations presented above.

***Findings from Chapter 3 have been published in the Journal of Vascular Surgery:***

Vun SV, Miller MD, Delaney CL, Allan RB, Spark JI. The effect of supervised exercise therapy for intermittent claudication on lower limb lean mass. *Journal of Vascular Surgery*. 2016;64(6):1763-1769.

# 5 THE EFFECT OF TREADMILL EXERCISE ON MICRORNA-92A IN RELATION TO ENDOTHELIAL FUNCTION AND WALKING PERFORMANCE

## 5.1 **Abstract**

*Objectives*

Little is known about miRNAs in PAD. Inhibition of pro-atherogenic, blood flow-responsive miR-92a has been shown to improve recovery and angiogenesis of muscles in animal models of lower limb ischaemia. Exercise is often prescribed to patients with PAD in the hope this increases limb perfusion and endothelial function, but the mechanism is unclear. The aim of this study was to ascertain the effect of exercise on the level of miR-92a in the serum and muscle of patients with PAD undergoing a SEP, and whether it is related to non-invasive measures of endothelial function. The primary hypothesis was that miR-92a levels would decrease as a result of the exercise intervention.

*Methods*

Patients with calf claudication were recruited into a 12-week SEP of twice-weekly treadmill walking. Endothelial function was assessed using brachial FMD and PAT. Pre- and post-intervention serum samples, and muscle biopsies from the symptomatic calf (ischaemic) and ipsilateral thigh (non-ischaemic) were obtained. miR-92a expression was quantified using TaqMan microRNA relative quantification RT-PCR assay.

*Results*

Twenty-three patients (mean age: 70.4 years; male: 17/23) completed the SEP. At baseline, miR-92a expression did not differ between calf vs. thigh (p=0.86). Following SEP, miR-92a expression significantly decreased in the thigh (p=0.04) but did not in the calf (p=0.13), while serum levels of miR-92a decreased significantly (p=0.004). A significant change in non-invasively measured endothelial function was not observed.

*Conclusions*

Exercise, possibly through local and systemic increases in shear stress, led to a significant decrease in muscle and circulating levels of miR-92a, which may be beneficial to overall endothelial health. Interventions that alter miR-92a levels may serve as useful targets in treating PAD and other cardiovascular diseases.

## 5.2 **Introduction**

Alterations to endothelial function are thought to be at the heart of the initiation and progression of atherosclerotic diseases such as peripheral artery disease (PAD) (Tabas, García-Cardeña & Owens, 2015). Endothelial dysfunction can be assessed non-invasively by flow mediated-dilatation (FMD) and peripheral artery tonometry (PAT) (Rubinshtein *et al.*, 2010) and these measurements are associated with disease progression, poor cardiovascular outcomes and premature death (Yeboah *et al.*, 2007). Many patients with PAD have demonstrable endothelial dysfunction (Brevetti, Schiano & Chiariello, 2008). Exercise improves endothelial function in healthy individuals (Vita, 2011), and there is evidence that exercise also improves endothelial function in patients with PAD (Januszek *et al.*, 2014). Exercise, however, in the presence of haemodynamically significant arterial lesions may cause disturbed or turbulent blood flow and a low-grade ischaemia-reperfusion stimulus that may worsen endothelial function in the long term (Turton *et al.*, 1998; Delaney *et al.*, 2014). Evidence regarding the impact of supervised exercise programs (SEP) on endothelial function is conflicting with some studies reporting improvement and others no change (McDermott *et al.*, 2009a; Delaney *et al.*, 2015). Differences between the methods used to assess endothelial function may account for this discrepancy and investigating other markers of endothelial function may help resolve the effect of SEP (Doshi *et al.*, 2001).

In the last decade there has been a large body of research into small non-coding ribonucleic acid (RNA) molecules, termed microRNAs (miRNAs), that regulate gene expression by translational repression or transcript degradation (Condorelli, Latronico & Cavarretta, 2014). Emerging evidence suggests that a number of miRNAs are important in the development of endothelial dysfunction and

atherogenesis and through large-scale profiling, candidate miRNAs that regulate vascular function have been discovered (Kumar *et al.*, 2014; Fichtlscherer *et al.*, 2011). One of these, miR-92a, has been shown to be up-regulated by low shear-stress or disturbed flow, promoting endothelial inflammation and atherosclerosis (Wu *et al.*, 2011; Loyer *et al.*, 2014). Furthermore, animal models of hind-limb ischaemia have demonstrated increases in muscle expression of miR-92a in response to disturbed flow and ischaemic injury (Bonauer *et al.*, 2009). Conversely, the administration of a pharmacological antagonist of miR-92a, has been shown to improve endothelial dysfunction, ischaemic recovery, and angiogenesis in animal models of ischaemia-reperfusion (Hinkel *et al.*, 2013a; Bonauer *et al.*, 2009; Loyer *et al.*, 2014). There have, however, been very few in vivo human studies on miR-92a and cardiovascular disease. It has been shown from microRNA profile studies that expression of baseline circulating miR-92a is significantly lower in patients with advanced coronary artery stenosis receiving state-of the-art pharmacological treatment versus healthy controls (Fichtlscherer *et al.*, 2010). There are, however, no data available with regard to miR-92a levels in patients with PAD, nor whether miR-92a levels can be altered by exercise in patients with symptomatic claudication, who are exposed to recurrent cycles of ischaemia-reperfusion as part of a SEP. Given the established role of miR-92a in endothelial function and atherogenesis, investigating the effect of SEP on miR-92a levels, in conjunction with FMD and PAT, will add crucial insight into the effects SEP has on endothelial function in patients with PAD.

As treadmill walking SEP is the recommended first line intervention for patients with intermittent claudication (IC), and one of its beneficial effects is presumed to occur through improvement in endothelial function and shear-stress, the aim of this study was to ascertain the effect of treadmill-based SEP on: **(1)** non-invasive

measures of endothelial function (FMD and PAT); **(2)** levels of miR-92a in symptomatic and asymptomatic muscles; **(3)** circulating miR-92a levels in serum of patients with IC undergoing 12-weeks of the currently recommended supervised exercise program (SEP). By utilising muscle biopsies from the vastus lateralis and gastrocnemius muscles of patients with calf claudication, the effect of exercise on the ischaemic calf can be compared with the normally perfused thigh muscle. Furthermore, serum was used as an additional tissue because it is readily collected and circulating miRNAs have been found to be remarkably stable in blood samples, even under unforgiving conditions such long-term storage, multiple freeze-thaw cycles, and extremes of pH and temperature (Mitchell *et al.*, 2008). It was hypothesised that 12-weeks of supervised exercise would improve shear-stress and thus endothelial function, evident by increases in FMD and PAT, and lower the levels of miR-92a in serum samples and leg muscle biopsies. The primary hypothesis was that miR-92a levels would decrease in muscle and serum samples as a result of the exercise intervention along with the other markers of endothelial function, FMD and PAT.

## 5.3 **Methods**

Refer to Chapter 3 for Common Methods: 3.1 Study Design and Setting, 3.2 Study Population and Eligibility, 3.3 Procedure for Informed Consent, 3.4 Exercise Intervention, 3.5.1 Routine Assessment, 3.5.3.1 Assessment of Exercise and Walking Performance.

### 5.3.1 **Micro-RNA-92a Levels in Serum and Muscle Biopsies**

#### 5.3.1.1 **Muscle Biopsies**

Muscle biopsy

Ultrasound was used to guide percutaneous skeletal muscle biopsies from symptomatic medial gastrocnemius muscle and ipsilateral vastus lateralis muscle. In cases where both legs were symptomatic, the leg with lowest resting ABPIs was chosen. After infiltration of the skin with 10-15mL of 1% lignocaine (Pfizer, West Ryde, NSW, Australia) a disposable co-axial biopsy needle (Bard TruGuide, Bard Peripheral Vascular, Tempe, Arizona, USA) was inserted into the muscle followed by deployment of 16-gauge disposable core biopsy instrument (Bard MaxCore, Bard Peripheral Vascular, Tempe, Arizona, USA). This was deployed 3-4 times to enable an adequate amount of skeletal muscle tissue to be collected (**Error! Reference source not found.**). Muscle samples were immediately placed in sterile tubes containing 2mL of proprietary RNA preservation buffer, RNA*later*® (Ambion, Foster City, CA, USA). Specimens were stored at 4 °C overnight, then at -80 °C after decanting excess RNA*later*®.

Figure 5.1. Photo demonstrating practice muscle biopsy technique prior to collection from study participants.

#### 5.3.1.2 **Blood Collection**

Blood testing

Participants were instructed to fast for 8-12 hours overnight and withhold morning medications. Blood was drawn via the arm veins using a Vacutainer system. Blood for microRNA quantification was collected into 8mL serum separator tubes, allowed to clot for 15 minutes, and centrifuged at 3,000g for 10 minutes. The serum was then aliquoted and stored immediately at -80°C until needed for total RNA extraction.

#### 5.3.1.3 **MicroRNA**

##### 5.3.1.3.1 **microRNA Isolation from Muscle**

RNA isolation from muscle

Total RNA was isolated by using an acid guanidinium thiocyanate-phenol-chloroform extraction miRNA isolation protocol originally developed by Chomczynski and Sacchi (Chomczynski & Sacchi, 1987, 2006). Skeletal muscle samples were first homogenised in sterile micro Eppendorf tubes by adding 500µL of TRIzol® (Invitrogen, Newcastle, NSW, Australia) using a sterile rotary polypropylene micro-pestle. TRIzol® is a proprietary solution of guanidinium thiocyanate and acid phenol which denatures protein enzymes and allows phase separation of RNA into an aqueous phase, and DNA and proteins into an organic phase. After a 5 minutes incubation at room temperature, 100µL of chloroform (Chem-supply, Gillman, SA, Australia) is added to facilitate phase separation and is then mixed well by shaking vigorously for 15 seconds. The samples were then incubated for 3 minutes at room temperature. Phase separation was then performed by centrifugation at 12,000 g for 15 minutes at 4 °C . The RNA containing aqueous phase (~140µL) was then carefully removed by micropipette, and placed in a new tube, to which 250µL of pure isopropanol (Chem-supply, Gillman, SA, Australia) was added to cause RNA precipitation. After a brief vortex, the sample was incubated for 10 minutes at room temperature, then centrifuged at 12,000 g for a further 10 minutes at 4°C allowing a gel-like pellet to form at the bottom of the tube. The supernatant was carefully removed and RNA pellet washed by adding 500µL of 75% ethanol (Chem-supply, Gillman, SA, Australia) and centrifuging at 7,500 g for 5 minutes at 4°C. The wash solution was carefully removed by micropipette and the pellet air dried placing the sample tube on dry ice for 4 minutes. The resultant pellet containing the purified RNA sample was then re-suspended in 15µL of sterile deionized RNase-free water

ready for quantification. This technique was practiced on banked human colonic and skeletal muscle samples prior to processing of study samples.

#### 5.3.1.3.2 **microRNA Isolation from Serum**

RNA isolation from serum

Total RNA from serum was isolated using a liquid specimen-optimised TRIzol-LS isolation protocol. TRIzol-LS differs from standard TRIzol in concentration allowing larger volume samples to be used up to 250µL. After thawing of serum samples, 250µL of sample serum was spiked with 2.5µL (0.02pmol/µL) of cel-miR-54 (Applied Biosystems, Foster City, CA, USA) and 750µL of Trizol-LS (Invitrogen, Newcastle, NSW, Australia) added and homogenized by vortex mixer. Spiking the samples with a known quantity of *Caenorhabditis elegans* cel-miR-54 permits the quantification and normalisation of the miRNA of interest. After a 5 minute incubation at room temperature, 200µL of chloroform was added to facilitate phase separation, mixed well and incubated for a further 3 minutes. Phase separation was then performed by centrifugation at 12,000g for 15 minutes at 4 °C . Subsequently, 400µL of the aqueous phase was removed, to which 2µL of UltraPure™ Glycogen (Invitrogen, Newcastle, NSW, Australia) and 500µL of pure isopropanol (Chem-supply, Gillman, SA, Australia) was added, mixed by inversion, and incubated overnight at -20°C to co-precipitate. Glycogen is The following day, the sample was then incubated at room temperature for 10 minutes then centrifuged at 12,000g for 10 minutes at 4 °C to pellet the precipitated RNA. The resultant RNA pellet was then washed by adding 1mL of ice cold 75% ethanol, vortexing briefly, then centrifuging at 7,500 for 5 minutes at 4°C. After

careful removal of the wash, the RNA pellet was dried at room temperature for 7 minutes. The dried pellet was then re-suspended in 20μL of sterile deionised RNase-free water ready for quantification.

#### 5.3.1.4 **microRNA Quantification**

##### 5.3.1.4.1 **RNA Quantification**

The isolated total RNA from the samples was then quantified using a Nanodrop-8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Nanodrop-8000 pedestals were first blanked with 1 μL water, then each sample was quantified by loading 1 μL onto the pedestal to obtain RNA quantity. The ratio of absorbance at 230nm, 260nm 280 nm was used to assess the purity of RNA accepting a 260/280 and 260/230 ratio >2.0 as "pure". Ratios <2.0 typically indicate contamination with protein, phenol or other contaminants. RNA was then diluted to a final concentration of 8ng/μL and stored at -80°C until ready for miRNA quantification by RT-PCR

##### 5.3.1.4.2 **Quantification of miRNAs**

Mature miRNAs were quantified by first performing reverse-transcription (RT) of the total RNA sample to cDNA, then performing real-time PCR on the resultant cDNA using TaqMan microRNA assay (Applied Biosystems, Foster City, CA, USA) protocol.

##### 5.3.1.4.3 **Reverse Transcriptase Reaction**

TaqMan MicroRNA Reverse Transcription Master Mix (Applied Biosystems, Foster City, CA, USA) was prepared on ice, by mixing 0.075 μL of 100mM deoxyribonucleotide triphosphate (dNTP) mix (with deoxythymidine triphosphate (dTTP)), 0.5 μL Multiscribe reverse transcriptase (50U/μL), 0.75μL of 10× reverse transcriptase buffer, 0.095 μL of RNase inhibitor (20U/μL), and 2.08 μL of nuclease-free water, for each sample. The RT reaction was then prepared, on ice, by adding 3.5 μL of the prepared Master Mix, 2.5 μL of the total RNA sample (8ng/μL), and 1.5 μL of stem–loop RT specific primer, to the wells of 8-strip PCR tubes. Samples were incubated on ice for 5 minutes, then loaded into a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The cycle was programmed for a 30-minute incubation at 16°C, followed by a 30-minute incubation at 42 °C , followed by a 5-minute incubation at 85°C, and then to hold at 4 °C until ready for use in real-time PCR.

#### 5.3.1.4.4 **Real-time PCR Reaction**

Real-time PCR was performed on the resultant cDNA, in triplicate, following the real-time PCR TaqMan protocol. To 4-strip PCR tubes, 1 μL of the sample cDNA was added together with 5 μL of 1× TaqMan Universal PCR Master Mix No AmpErase® UNG, 3.84μL of nuclease-free water, and 0.5 μL of TaqMan miRNA-specific primer/probe assay mix (**Error! Reference source not found.**). Tubes were then loaded onto a Qiagen Rotor-Gene Q (Qiagen, Valencia, CA, USA) thermal cycler programmed for 10-minute incubation at 95°C, then 50 cycles of a 15 second denaturing step at 95°C and a 60 second annealing/extension step at 60°C.

Reactions also included the positive control (colonic tissue 100B), a negative control tube with no RNA sample to control for contamination, and a five-fold serial dilution of the input RNA sample (undiluted, 1:4, 1:16, 1:64, 1:256) to create a standard curve to determine the amplification efficiency.

MiRNA levels were normalised relative to the levels of the endogenous small nuclear RNA gene RNU6B in the case of muscle samples, and cel-miR-54 in the case of serum samples. Expression levels were calculated from Ct values using Q-gene (Muller *et al.*, 2002).

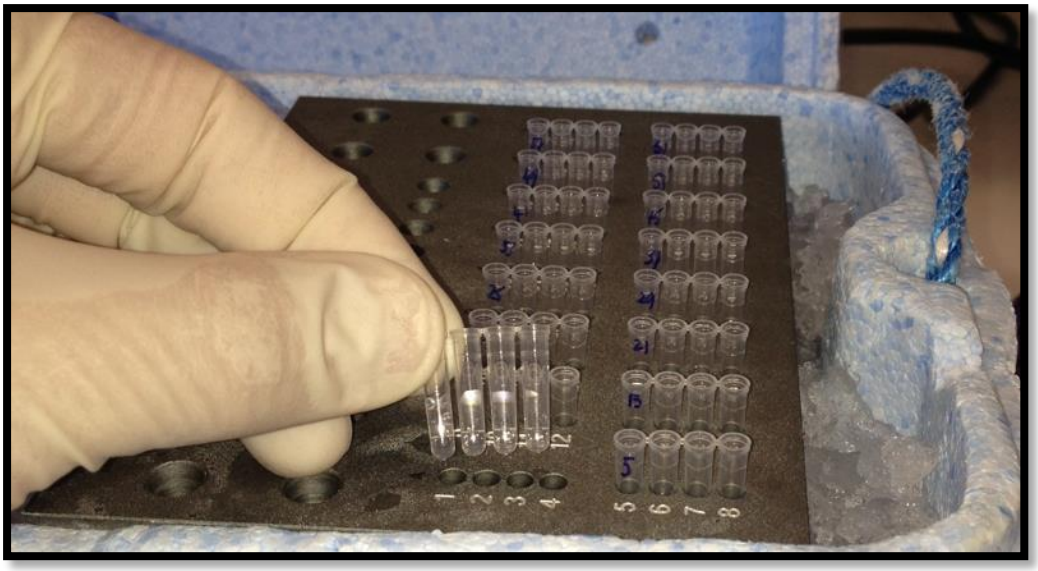

Figure 5.2. Preparation of the real-time PCR reaction into 4-strip PCR tubes on ice.

### 5.3.3 **Measures of Endothelial Function**

Refer to Chapter 3.5.3.2

### 5.3.4 **Statistical Analysis**

Continuous variables were assessed using D'Agostino and Pearson's omnibus normality testing. Variables passing normality testing are expressed as mean ± standard deviation, and those not passing are expressed as median ± interquartile range. Between-group comparisons were made using the Student's *t*-test, or Mann-Whitney *U* test when normality not assumed based on the outcome of normality testing. Pair-wise comparisons were made using the Student's paired *t* test, or the Wilcoxon matched-pairs signed rank test, where normality was not assumed based on the outcome of normality testing. Pearson correlation analysis was used to evaluate the relationship between study variables of endothelial function. A post-hoc analysis of miR -92a levels was performed to compare miR-92a levels between patients SEP responders vs. non-responders, bilateral vs. unilateral disease, and between patients with occlusive versus stenotic arterial disease.

#### 5.3.4.1 **Sample Size Justification**

For the primary outcome of miR-92a level in muscle, sample size was calculated based on the assumption of an effect size (Cohen's d) of 0.54 (mean of differences = 120 mean normalised expression, SD of the difference = 230 mean normalised expression). Using G*Power software version 3.1 for OSX (Düsseldorf, Germany), the sample size for a two-sided Wilcoxon signed-rank test (matched pairs) was calculated *a priori*. Assuming an effect size of 0.54, $\alpha = 0.05$, and $\beta = 0.80$, a total sample size of 30 pairs would be required.

## 5.4 **Results**

The included patients are described in Section 0. The patients included in the miRNA analysis are a subset of the group in the recruited in the previous chapter. MicroRNA data was not available for two patients owing to a poor recovery of RNA from tissue samples. Participant characteristics at entry to the study are shown for the remaining twenty-three patients included in this analysis in Table 5.1. Blood analysis results of the patients at baseline are also shown Table 5.2. The study outcomes are shown in Table 5.3. Walking outcomes, PFWD and 6MWD, significantly improved following the 12-week supervised exercise program (mean, 39 (SD, 76) m; p=0.03) and (mean, 30 (SD, 44) m; p=0.003) respectively. In contrast, no significant change in measures of endothelial function, RHI and FMD, were observed.

At baseline, muscle levels of miRNA-92a expression did not differ between calf and the thigh (median, 286 (IQR, 140-455) vs. 270 (IQR 123-406); p=0.93) (Table 5.3, Figure 5.3). However, following the supervised exercise program, miR-92a levels significantly decreased in the thigh (median, 270 (IQR 123-406) vs. 141 (IQR 102-276); (p=0.04) but did not in the calf (median, 286 (IQR, 140-455) vs. 200 (IQR 108-287); p=0.13). Furthermore, levels of miR-92a from serum samples decreased significantly follow the exercise program (median, 1.52 (IQR, 0.91-1.89) vs. 0.87 (IQR 0.70-1.25); p=0.004).

micro-RNA-92a levels in both muscle and serum samples did not significantly differ between groups with bilateral vs. unilateral disease, nor between groups with occlusive versus stenotic disease. No significant correlation was observed

between changes in any of the various measures of endothelial function, microRNA-92a, or walking performance: (PFWD vs. serum miR-92a r=0.05, p=0.84), (6MWD vs. serum miR-92a r=-.36, p=0.14), (PFWD vs. calf miR-92a r=0.3, p=0.19), (PFWD vs. thigh miR-92a r=0.11, p=0.63, (6MWD vs. thigh miR-92a r=0.04, p=0.83), (FMD vs. serum miR-92a r=-0.3, p=0.23), (FMD vs. calf miR-92a r=-0.3, p=0.14), (FMD vs. thigh miR-92a r=0.09, p=0.67), (RHI vs. serum miR-92a r=-0.27, p=0.31), (RHI vs. calf miR-92a r=0.11, p=0.61), (RHI vs. thigh miR-92a r=-0.05, p=0.84).

Table 5.1. Participant characteristics at baseline of patients undergoing measurement endothelial function and microRNA-92a.

| **Variable** | **Result** | |
|---|---|---|
| No. | 23 | |
| Age, mean (SD), years | 70.4 | (10) |
| Sex | | |
| Male, (%) | 17 | (74) |
| Female, (%) | 6 | (26) |
| BMI mean (SD), $kg/m^2$ | 27.4 | (4) |
| Current smokers, No. (%) | 10 | (43) |
| Former smokers, No. (%) | 12 | (52) |
| Pack-years, mean (SD) | 40.29 | (24) |
| Diabetics, No. (%) | 6 | (26) |
| Hypertension, No. (%) | 19 | (83) |
| Dyslipidaemia | 20 | (87) |
| Ischaemic heart disease, No. (%) | 6 | (26) |
| AAA, No. (%) | 3 | (13) |
| COPD, No. (%) | 1 | (4) |
| ABPI, mean (SD) | | |
| Lowest resting | 0.71 | (0.2) |
| Lowest post-exercise | 0.32 | (0.2) |
| Medications | | |
| ACE-I/ARA, No. (%) | 17 | (74) |
| Beta-blockers, No. (%) | 7 | (30) |
| Aspirin, No. (%) | 18 | (78) |
| Clopidogrel, No. (%) | 3 | (13) |
| Statins, No. (%) | 18 | (78) |

AAA, Abdominal aortic aneurysm; ABPI, ankle-brachial index; ACE-I, angiotensin-converting enzyme inhibitor; ARA, angiotensin receptor antagonist; BMI, body mass index; COPD, chronic obstructive pulmonary disease; SD, standard deviation.

Table 5.2. Laboratory values at baseline of patients undergoing measurement endothelial function and microRNA-92a.

| **Variable** | **Mean** | **SD** | **Ref Range** |
|---|---|---|---|
| No. | 23 | | |
| Haemoglobin, g/L | 141 | (14) | ♂: 130-170<br>♀: 120-150 |
| White cells, $x10^9$/L | 7.7 | (2.3) | 4-10 |
| Neutrophils, $x10^9$/L | 5.2 | (1.9) | 2-7 |
| C-reactive protein, mg/L | 8.0 | (29) | <5 |
| Creatinine, μmol/L | 79 | (22) | ♂: 60-110<br>♀: 45-90 |
| Albumin, g/L | 41 | (4) | 33-48 |
| Total cholesterol, mmol/L | 3.9 | (1.2) | <4.0 |
| HDL, mmol/L | 1.4 | (0.5) | 1.0-2.2 |
| LDL, mmol/L | 2.1 | (1.0) | 2.0-3.4 |
| Triglycerides, mmol/L | 1.5 | (0.7) | <2.0 |
| Homocysteine, μmol/L | 13 | (4.2) | 5-15 |
| $HbA_1c$ (%) | 6.1 | (0.8) | 3.5-6 |

HbA1c, Glycosylated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

Table 5.3. Study outcomes of patients undergoing measurement endothelial function and microRNA-92a.

| **Variable** | **Baseline** | | **12-weeks** | | **Change** | | **p-value** |
|---|---|---|---|---|---|---|---|
| PFWD, mean (SD), m | 175.5 | (87.3) | 217.9 | (106.7) | 39.4 | (39) | 0.03 |
| 6MWD, mean (SD), m | 393.4 | (68.6) | 423.4 | (75.89) | 29.9 | (30) | 0.003 |
| RHI, mean (SD) | 2.11 | (0.61) | 2.09 | (0.66) | -0.1 | (0.5) | 0.37 |
| FMD, mean (SD), % | 3.511 | (2.7) | 3.530 | (2.685) | 0.3 | (3.4) | 0.69 |
| miRNA-92a expression | | | | | | | |
| Serum, median (IQR) | 1.52 | (0.91-1.89) | 0.87 | (0.70-1.25) | -0.5 | | 0.004 |
| Muscle | | | | | | | |
| Calf, median (IQR) | 286 | (140-455) | 200 | (108-287) | -15 | | 0.13 |
| Thigh, median (IQR) | 270 | (123-406) | 141 | (102-276) | -41 | | 0.04 |

6MWD, 6-minute walk distance; FMD, flow mediated dilation; PFWD, pain-free walking distance; RHI, reactive hyperaemia index; SD, standard deviation;

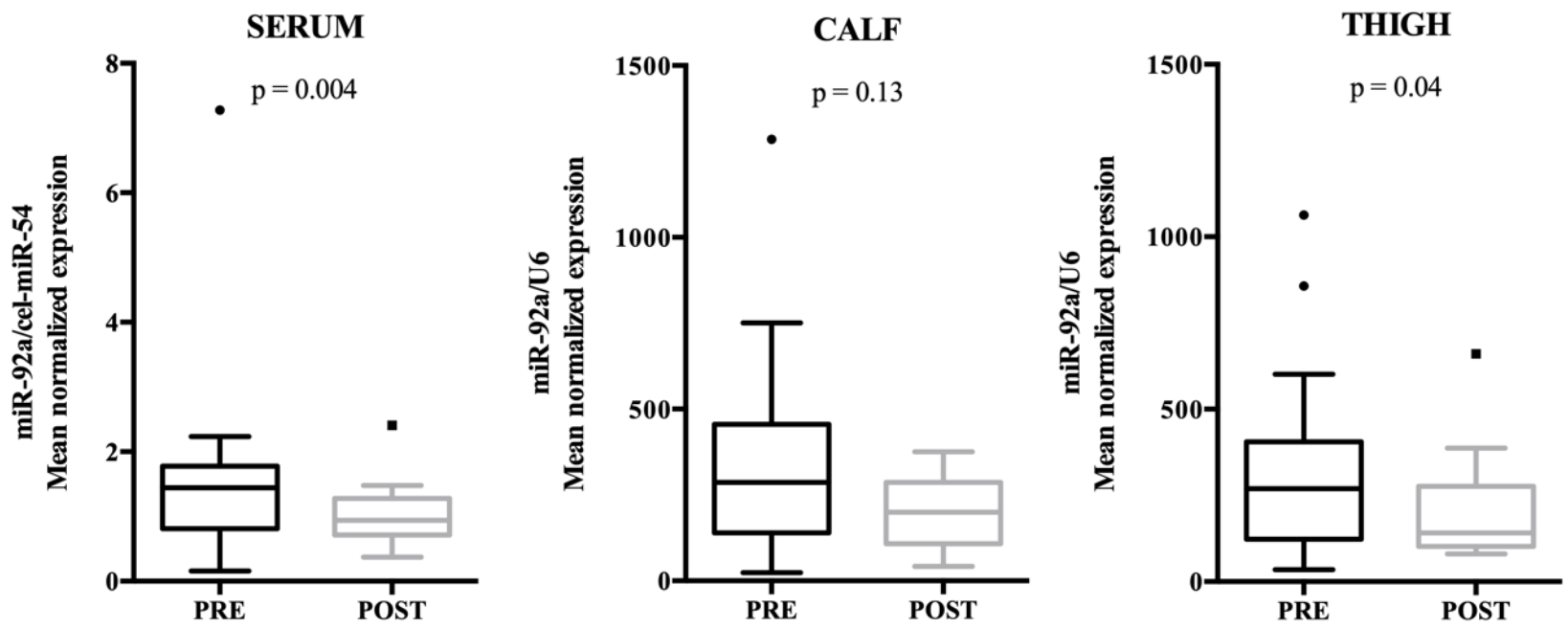


Figure 5.3. Pre- and post- supervised exercise training microRNA levels in serum and muscle biopsies from patients with claudication at baseline (Wilcoxon matched-pairs signed rank test)

## 5.5 **Discussion**

Intermittent claudication remains a frequent problem encountered by vascular surgeons and physicians with over 202 million people affected by PAD worldwide (Fowkes *et al.*, 2013). Whilst exercise has been shown to improve walking performance in many studies (Lane *et al.*, 2017; Hageman *et al.*, 2018), evidence demonstrating improved survival is surprisingly scarce. Despite the first randomised control trials of exercise being performed nearly 50 years ago almost none reported cardiovascular or survival outcomes. To date only one retrospective study of non-randomised patients assessed cardiovascular events and survival outcomes (Sakamoto *et al.*, 2009). Indeed some authors have previously speculated that exercise in the setting of haemodynamic compromise may be harmful due to the effects of recurrent ischaemia-reperfusion (Turton *et al.*, 1998; Delaney *et al.*, 2014). Despite this, the evidence that SEP improves walking performance is very strong and results from the current study support the concept that 12-weeks of supervised treadmill-based walking exercise can improve PFWD and 6MWD.

Unique to this study are findings regarding the impact of SEP on miR-92a levels. Elevated levels of miR-92a appear to promote atherosclerosis and endothelial dysfunction (Loyer *et al.*, 2014), which are up-regulated by disturbed flow or oscillatory shear stress and down-regulated by laminar shear stress (Wu *et al.*, 2011; Fang & Davies, 2012). In this study, a 12-week SEP was associated with a decrease in miR-92a in serum and asymptomatic thigh muscle and a non-significant trend towards lower miR-92a levels in symptomatic calf muscle. These changes in miR-92a levels in serum and thigh muscle may be due to an increase in local and whole-body laminar blood flow shear stress as a result of the aerobic

exercise and the increases in cardiac output that accompany walking exercise. It is therefore conceivable that SEP may impart a positive effect on systemic endothelial health via this mechanism.

With respect to the ischaemic calf muscles associated with infra-inguinal arterial disease, the presence of localised haemodynamic compromise and subsequent impairment of laminar blood flow, possibly blunted the effect on miR-92a expression resulting in a non-significant trend to lower levels. It is conceivable that a combination of corrected haemodynamics, achieved through revascularisation, and exercise associated increases in blood flow from SEP would be synergistic and result in a statistically significant downregulation of miR-92a levels in previously ischaemic muscles. Local improvement in blood flow and shear-stress related miR-92a level achieved through durable revascularisation and exercise, may result in improved muscular function and recovery. Interestingly, in a porcine model of ischaemia-reperfusion, local inhibition of miR-92a, rather than systemic delivery of a miR-92a antagonist resulted in improved end-organ functional outcomes such as reduced myocardial ischaemic injury and improved contractile function (Hinkel *et al.*, 2013b). Moreover, there is recent evidence that pharmacological inhibition of miR-92a improves endothelial healing and attenuates neointimal hyperplasia following endothelial injury (Daniel *et al.*, 2014). Together, these results are consistent with recent data from randomised clinical trials where the combination of endovascular revascularisation and SEP resulted in greater improvements in walking performance together with reduced rates of restenosis and reintervention, compared to either revascularisation or SEP alone (Mazari *et al.*, 2017; Fakhry *et al.*, 2015).

It was interesting to note there was no correlation between the walking endpoints (PFWD and 6MWD), miRNA-92a and the other markers of endothelial function. The absence of correlation, however, does not imply an absence of a relationship. Furthermore, it is possible that the study sample lacked sufficient statistical power or range of variation, which may have prevented the detection of a correlation. A larger study that accounts for confounding factors and includes patients across the PAD spectrum along with healthy matched controls can address these limitations. The results of the current study have additional important clinical implications given the role of miR-92a in angiogenesis, vascular inflammation, and functional recovery in ischaemic tissues (Bonauer *et al.*, 2009). It has been previously demonstrated that miR-92a has an important role in endothelial activation and dysfunction (de Winther & Lutgens, 2014). Recently, it was shown that the uremic toxins that accumulate due to end-stage renal failure upregulate miR-92a levels, which likely predispose these patients to cardiovascular events and premature death (Shang *et al.*, 2017). Furthermore, Zhang *et al*. demonstrated that circulating miR-92a levels were significantly higher in patients suffering acute myocardial infarction (AMI) compared to patients with stable coronary artery disease and serum miR-92a levels demonstrated good diagnostic performance on receiver-operating characteristic curve analysis (AUC= 0.89)(Zhang *et al.*, 2017). Similarly, Wang *et al*. reported significantly higher serum levels of miR-92a in patients with acute coronary syndrome compared to stable CAD, particularly in those who were diabetic (Wang *et al.*, 2019). Collectively, this suggests that high levels of circulating miR-92a is a biomarker for endothelial activation and risk of future cardiovascular events.

Given the link between miR-92a levels and vascular health, it could be speculated that non-invasive markers of endothelial function (FMD and PAT) would improve

in accordance with miR-92a levels. This was not the case in the current study, however, which is in contrast to work from other studies with reported improvement in FMD in similar cohorts of patients undergoing exercise therapy (McDermott *et al.*, 2009a; Januszek *et al.*, 2014; Mika *et al.*, 2013). There are several possible explanations for this. Firstly, a major methodological difference between studies needs consideration. Previous studies have used a proximal arm occlusion method that has subsequently been shown to result from endothelium-independent responses such as myogenic and sympathetic reflexes (Berry, Skyrme-Jones & Meredith, 2000; Doshi *et al.*, 2001). Such responses may lead to an overestimation of endothelial-dependent vasodilation or reflect non-endothelial adaptation of uncertain significance. In this study, a forearm occlusion method was used, which is now recognised to provide endothelium-dependent results only (Doshi *et al.*, 2001). Peripheral artery tonometry, on the other hand, assesses digital reactive hyperaemia, which appears to be dependent on changes in the microcirculation and, to a lesser extent, conduit arteries and endothelial-dependent relaxation (Nohria, 2006). Interestingly, the mean RHI for this cohort was >2, which is above manufacturers recommended cut-off for endothelial dysfunction. This is surprising given that endothelial dysfunction is thought to precede the onset of atherosclerosis, however, similar findings in PAD have been reported before (Allan *et al.*, 2013) and may be reflective of the fact that nearly all patients were taking optimum secondary prevention medication including HMG-CoA-reductase inhibitors, which have been shown to improve endothelial function (O'Driscoll, Green & Taylor, 1997). Even though patients withheld their morning medications, it is possible that the beneficial effects of statin medication persisted into the testing period. It should also be mentioned that this pilot study was designed to explore muscle and serum levels of miR-92a. Due to the limited

sample size, there may have been insufficient statistical power to detect changes in non-invasive markers of endothelial function Indeed, based on the sample size calculation carried out for the primary outcome, this study was underpowered, thus the non-significant trend in thigh miR-92a levels may represent type II error.

In summary, this study is the first to investigate the effect of SEP on miR-92a levels in humans with peripheral arterial disease. The unique findings demonstrate a down-regulation in miR-92a levels which may be anti-atherogenic and beneficial to the health of the vascular endothelium. This challenges previous concerns that have been raised regarding the safety of exercise training in patients with IC. Intervening to downregulate miR-92a either through exercise, pharmacological or durable surgical means, may have positive clinical effects by reducing endothelial activation, restenosis/reintervention rates, and in turn cardiovascular morbidity and mortality. In addition, miR-92a may serve as clinically useful as a biomarker for vascular health and future cardiovascular risk. More large-scale controlled studies with long term follow-up are needed to learn more about the true potential of this miRNA.

# 6 THE EFFECT OF TREADMILL EXERCISE ON CIRCULATING SUB-POPULATIONS OF MONOCYTES

## 6.1 **Abstract**

*Objectives*

Monocytes are chronic elements of the innate immune system which have a well-known role in plaque development and its complications. The ability to modify the chronic inflammatory nature of PAD through exercise may improve clinical outcomes. However, the chronic low-grade exposure to an ischaemia-reperfusion environment may have unexpected effects on monocyte numbers and function. The aim of this study was to measure the effect of supervised exercise on pro-inflammatory monocyte subtypes and CD16 receptor expression.

*Methods*

Patients with calf claudication were recruited into a standard treadmill based supervised exercise program. Before and at the completion of a 12-week exercise program, patients had peripheral blood drawn to analyse monocyte subtypes using flow-cytometric methods. In addition, a convenience sample of healthy controls was included for comparison at baseline.

*Results*

Twenty-six patients with claudication and twenty healthy controls had monocyte analysis completed at baseline. Patients had higher levels of inflammatory markers CRP and WCC, but also greater total monocyte count (p=0.01) and numbers of pro-inflammatory intermediate monocytes (p=0.03). Additionally, monocyte CD16 expression was significantly greater in patients vs. controls (p=0.009). Following the exercise program, there was no change detected in numbers or proportions of

monocyte subtypes, however, monocyte CD16 expression decreased towards that of healthy controls (p=0.03).

*Conclusions*

Patients with intermittent claudication appear to have greater numbers of total and pro-inflammatory monocytes, compared to controls, however, 12-weeks of supervised treadmill exercise does not appear to increase or decrease either measure. In addition, the monocytes of patients appear to express greater levels of CD16, compared to controls, and this decreased following 12-weeks of supervised treadmill exercise suggesting an anti-inflammatory adaptation.

## 6.2 **Introduction**

As a disease of chronic inflammation, the immune system is recognised as a key player in the development and progression of atherosclerotic disease such as PAD and claudication. Associations between humoral immune factors, such as hs-CRP and IL-6, and disease severity and progression have been demonstrated (Brevetti *et al.*, 2010). Likewise, acute cellular components of inflammation, such as neutrophils, are higher in patients with PAD compared to healthy matched controls and a higher neutrophil-to-lymphocyte ratio predicts adverse outcomes (Chan *et al.*, 2014). On the other hand, chronic inflammatory elements of the immune system such as monocytes only account for about 5-10% of circulating white blood cells but are abundant in atherosclerotic plaques and have a demonstrated role in atherogenesis (Tabas & Lichtman, 2017). However, early studies on peripheral monocyte levels did not show an association with atherosclerotic disease or complications (Wheeler *et al.*, 2004). It has been postulated that this is due to the fact that circulating monocytes display considerable heterogeneity in phenotype and it has been recognised that monocytes exist in at least three distinct subset based on their immune-phenotype characterisation by flow cytometry (Passlick, Flieger & Ziegler-Heitbrock, 1989; Ziegler-Heitbrock *et al.*, 2010). The so-called "classical" monocytes characterised by high levels of the lipopolysaccharide (LPS) co-receptor CD14 and little to no expression of the Fcγ-III receptor CD16. Classical monocytes are the most abundant in peripheral blood and appear to have a predominantly phagocytic function with little or no pro-inflammatory attributes (Zawada *et al.*, 2011, 2012). On the other hand, the least abundant "intermediate" monocytes are characterised by CD14 expression but demonstrate increased expression of CD16 and are characterised by MHC II antigen processing and presentation to CD4 T-cells

(Rossol *et al.*, 2012), high basal ROS production (Zawada *et al.*, 2011), and simulate TNF-α and IL-1β production (Cros *et al.*, 2010; Zawada *et al.*, 2012). Non-classical monocytes are defined by very low expression of CD14 and very high expression of CD16 (Ziegler-Heitbrock *et al.*, 2010).

A number of recent studies have demonstrated clear associations between monocyte subpopulations and atherosclerotic disease. For example, high levels of intermediate monocytes appear to independently predict adverse cardiovascular events in patients undergoing elective coronary angiography (Rogacev *et al.*, 2012). In PAD, significantly higher proportions of intermediate monocytes are found in patients with claudication or critical limb ischaemia (Dopheide *et al.*, 2012). Endovascular revascularisation does not appear to affect their abundance (Maga *et al.*, 2016).

Monocyte subpopulations thus represent a potential therapeutic target to modify disease progression through either pharmacological or non-pharmacological means. Additionally, physical activity is widely believed to associated with a reduced inflammatory burden compared to a sedentary lifestyle (Beavers, Brinkley & Nicklas, 2010). Strenuous anaerobic exercise has been shown to lead to a significant increase in the circulating levels of $CD14^{+}/CD16^{+}$ monocytes (Simpson *et al.*, 2009; Steppich *et al.*, 2000). Conversely, regular submaximal aerobic exercise training has demonstrated anti-inflammatory ability (Timmerman *et al.*, 2008). With regard to monocyte subpopulations physical inactivity at baseline has been demonstrated to be associated with an elevated proportion of monocytes expressing CD16, and 12-weeks of aerobic training was shown to significantly reduce the percentage of peripheral monocytes expressing CD16

(Timmerman *et al.*, 2008). In PAD, however, very little is known about the effect of exercise on monocyte subpopulations and it's possible that regular exercise has a similar effect in reducing the proportion of inflammatory CD16 expressing monocytes. Conversely, the repeated episodes of ischaemia-reperfusion that is experienced by patients with claudication may promote a shift towards increased pro-inflammatory intermediate monocytes, which may ultimately have a negative impact on vascular health despite improvements in walking performance. As supervised treadmill exercise is the recommended first-line treatment for claudication, a supervised exercise program provides an opportunity to assess the circulating subpopulations of monocytes in patients with claudication and their response to 12-weeks of supervised treadmill-based exercise therapy. The primary aim of this study was to compare the relative proportions of monocyte subpopulations in patients with calf claudication to healthy controls, and to assess the effect of supervised exercise on the proportions of monocyte subpopulations in patients with calf claudication. The secondary aim was to measure monocyte CD16 expression on monocytes in patients and controls, and in patients following the exercise program. The primary hypothesis was that pro-inflammatory intermediate monocyte percentage would decrease following the exercise intervention.

## 6.3 **Methods**

Refer to Chapter 3 for Common Methods: 3.1 Study Design and Setting, 3.2 Study Population and Eligibility, 3.3 Procedure for Informed Consent, 3.4 Exercise Intervention, 3.5.1 Routine Assessment, 3.5.3.1 Assessment of Exercise and Walking Performance.

### 6.3.1 Circulating Monocyte Subpopulations

#### 6.3.1.1 Preparation of Whole Blood Assay

To analyse the circulating subpopulations of monocytes, 6mL of whole blood was drawn from each participant at the time of research assessment into lithium heparin collection tubes (Becton Dickinson, North Ryde, Australia). Blood samples were immediately transported to the flow cytometry laboratory for analysis. Fifty microliters of the whole blood sample were then added to 5 ml FACS tubes (BD Falcon, San Diego, CA, USA) together with 5μL of each fluorochrome-conjugated monoclonal antibody which allows detection during flow cytometry: CD45-Peridinin Chlorophyll Protein Complex (PerCP) cat no. 557513, CD86-Brilliant Violet™421 (BV421) cat no. 562432, CD14-Fluorescein isothiocyanate (FITC) cat no. 555397, and CD16-allophycocyanin (APC) cat no. 561248, (BD Biosciences, Wembley, Western Australia, Australia). Samples were then incubated in darkness for 20 minutes at room temperature. Erythrocytes were then lysed by adding 2mL of BD FACS™ Lysing Solution (cat no. 349202, BD Biosciences, Wembley, Western Australia, Australia) and incubated in darkness for 5 minutes at room temperature. Samples were then centrifuged at 500g for 5min and the supernatant tipped off. The samples were then washed by adding 2mL of wash buffer (1× phosphate-buffered saline + 1% foetal calf serum + 0.02% azide), centrifuging at 500g for 3min, then tipping off the supernatant. Washing was performed twice. The stained and washed cells were then resuspended in 500μL of 1× BD™ Stabilizing Fixative (cat no. 338036, BD Biosciences, Wembley, Western Australia, Australia), centrifuged at 500g for 3min. The supernatant was then tipped off and the samples assayed on a BD

FACSCanto II flow cytometer (BD Biosciences) counting a total of 100,000 cells. The data was then exported and analysed using FlowJo® software version 10.4.2 (Tree Star Inc., Oregon, USA) as below.

#### 6.3.1.2 **Four Colour Flow Cytometry for Monocyte Subpopulations**

Flow cytometric analysis was performed according to the gating strategy published by Dopheide *et al*. with slight modifications (Dopheide *et al.*, 2012) (**Error! Reference source not found.**). The morphology of cells was used to identify the monocyte population on the forward scatter (FSC) and side scatter (SSC) plot. Next, the selection of the monocyte population was further refined by only gating on cells bearing CD45 (leucocyte common antigen) and CD86 (pan-monocytic marker) to exclude other CD16-expressing leukocytes such as neutrophils and natural killer cells. From this population, monocyte subsets were defined according to the surface expression pattern monocytes of the CD14 (LPS receptor) and the Fcγ-III receptor CD16: classical monocytes were defined as $CD14^{++}/CD16^{-}$, intermediate monocytes $CD14^{++}/CD16^{+}$, non-classical monocytes $CD14^{+}/CD16^{++}$ according to the consensus published by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock *et al.*, 2010). See Figure 5.1. The geometric mean of fluorescence intensity was taken as the mean fluorescence intensity (MFI).

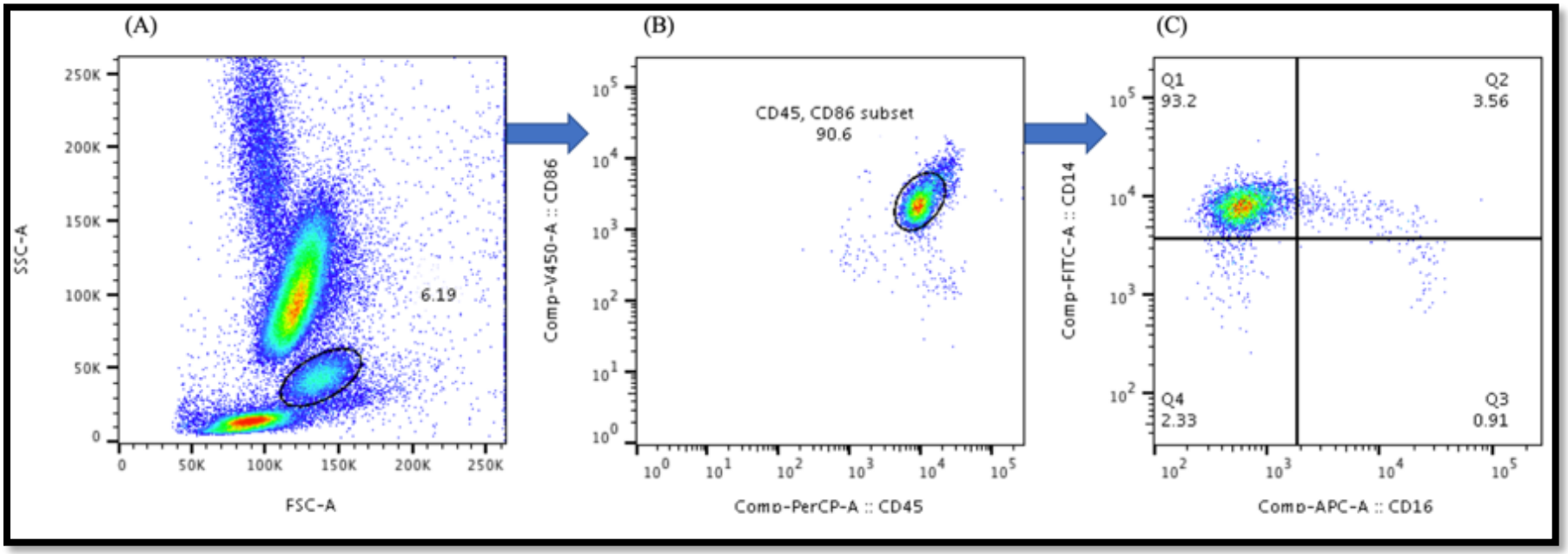
(A)
SSC-A
FSC-A
6.19
(B)
Comp-V450-A :: CD86
Comp-PerCP-A :: CD45
CD45, CD86 subset
90.6
(C)
Comp-FITC-A :: CD14
Comp-APC-A :: CD16
Q1
93.2
Q2
3.56
Q4
2.33
Q3
0.91

Figure 6.1. Gating strategy for analysis of monocyte subpopulations. Monocytes were initially identified based on the physical characteristics of size and granularity reflected by the forward scatter and side scatter plots, respectively (A). To further exclude neutrophils and NK cells, the initial population of monocytes were further selected based on CD45 and CD86 surface expression (B). From this population, monocyte subpopulations were analysed based on relative CD14 and CD16 expression (C).

Participants acting as non-diseased controls were recruited from the hospital volunteer's organisation and members of staff from the department of vascular surgery. All control participants underwent screening to exclude PAD though history and ABPI testing.

### 6.3.2 **Measures of Endothelial Function**

Refer to Chapter 3.5.3.2

### 6.3.3 **Statistical analysis**

Continuous variables were assessed using D'Agostino and Pearson's omnibus normality testing. Variables passing normality testing are expressed as mean ± standard deviation, and those not passing are expressed as median ± interquartile range. Between-group comparisons were made using the student's t test, or Mann-Whitney *U* test when normality not assumed. Within-group comparisons were compared using Wilcoxon matched-pairs signed rank test.

#### 6.3.3.1 **Sample Size Justification**

For the primary outcome of intermediate monocyte percentage, sample size was calculated based on the assumption of an effect size (Cohen's d) of 0.89 based on results previously reported by Timmerman *et al*. (mean pre-exercise = 13.2% (SD 10.45), mean % post exercise = 4.9% (SD 2.5) (Timmerman *et al.*, 2008). Using G*Power software version 3.1 for OSX (Düsseldorf, Germany), the sample size for a two-sided Wilcoxon signed-rank test (matched pairs) was calculated *a priori*. Assuming an effect size of 0.89, $\alpha = 0.05$, and $\beta = 0.80$, a total sample size of 14 pairs would be required.

## 6.4 **Results**

The patients included in the monocyte analysis are a subset of the group in the recruited in the previous Section 0. Flow cytometry data was not available for five of the first participants as the protocol had not yet been finalised. Twenty healthy control participants were recruited for analysis of monocytes, but they did not undergo exercise training. Due to unforeseen time and recruitment constraints, it was not possible to match controls to patient group. Table 6.1 outlines the baseline characteristics of the study sample. Patients with claudication were approximately 10-years older and were more likely to be male. In addition, patients had significantly higher BMI, serum creatinine, homocysteine, triglycerides, and HbA1c. Conversely, patients had significantly lower serum total cholesterol and LDL-c levels. Patients with claudication also had significantly higher conventional markers of inflammation such as, total WCC, neutrophils, total monocytes and serum CRP.

Classical ($CD14^{++}/CD16^{-}$) monocytes were the dominant population of circulating monocytes in both patients and healthy controls accounting for approximately 90% of total circulating monocytes (median ± IQR: 89.45 (85.43-92.43) %) (Figure 6.3). There was a trend towards higher absolute numbers of classical monocytes in patients, and this nearly reached statistical significance (0.44 (0.35-0.64) vs. 0.37 (0.30-0.42) x $10^9$/L; p=0.052) (Figure 6.2). Additionally, there was a statistically significantly higher absolute count of intermediate ($CD14^{+}/CD16^{++}$) monocytes (0.037 (0.026-0.071) vs. 0.021 (0.016-0.041) x $10^9$/L; p=0.03) (Figure 6.2). However, there was no significant difference in the percentage of each of the

three subtypes of monocytes (Figure 6.3). Monocyte expression of CD16, however, was statistically significantly higher in patients with claudication versus controls (median ± IQR: 1211 (980-1353) vs. 954 (850-1087) a.u; p=0.009) (Table 6.1, Figure 6.4).

Walking performance improved significantly at the 12-week follow-up testing (Table 6.2). Of the twenty patients that were able to participate in and complete the SEP there was not a significant difference between the absolute numbers or percentage of each of the monocyte subtypes at baseline or after completion of the exercise program (Table 6.3). There was, however, a significant decrease in the monocyte expression of CD16 (median ± IQR: 1218 (1013-1414) vs. 1066 (917-1226) a.u; p=0.03) (Figure 6.4).

There was no significant difference between pre and post exercise WCC (7.6 (2.1) x $10^9$/L vs. 7.8 (2.6) x $10^9$/L , p=0.42), neutrophil count (5.1 (1.4) x $10^9$/L vs. 5.1 (2.0) x $10^9$/L , p=0.95), total monocyte count (0.56 (0.24) x $10^9$/L vs. 0.60 (0.27) x $10^9$/L , p=0.23), , or CRP levels (2.2 (0.63-3.4) mg/L vs. 4.2 (0.56-3.6) mg/L , p=0.87).

Table 6.1. Demographic features and laboratory findings in patients with claudication and in controls undergoing measurement of monocyte subtypes.

| **Variable**<br>**No.** | **Patients with claudication (n=25)** | **Controls (n=20)** | **p-value** | **Ref Range** |
|---|---|---|---|---|
| Age, years (SD) | 67.9 (11.7) | 59.6 (8.2) | 0.009 | |
| Sex (M/F) | 21/5 | 8/13 | 0.006 | |
| BMI, $kg/m^2$ (SD) | 28.3 (4.8) | 25.9 (2.9) | 0.048 | |
| Creatinine, μmol/L (IQR) | 75.0 (66.75-90.50) | 70 (60.50-85.00) | 0.01 | ♂: 60-110<br>♀: 45-90 |
| Homocysteine, μmol/L (IQR) | 13.20 (10.18-15.03) | 9.8 (8.35-11.90) | 0.0006 | 5-15 |
| Triglycerides, mmol/L (IQR) | 1.40 (0.975-2.375) | 0.80 (0.60-1.00) | <0.0001 | <2.0 |
| Total cholesterol, mmol/L (SD) | 4.0 (1.2) | 5.1 (1.1) | 0.003 | <4.0 |
| LDL, mmol/L (SD) | 2.1 (0.97) | 2.8 (1.0) | 0.029 | 2.0-3.4 |
| HDL, mmol/L (IQR) | 1.3 (0.975-1.60) | 2.0 (1.55-2.30) | <0.0001 | 1.0-2.2 |
| HbA1c, % (IQR) | 5.8 (5.375-6.625) | 5.3 (4.90-5.55) | 0.001 | 3.5-6 |
| Haemoglobin, g/dL (IQR) | 140 (130.5-153.3) | 138 (131.5-148) | 0.40 | ♂: 130-170<br>♀: 120-150 |
| WCC, x $10^9$/L (SD) | 7.78 (2.1) | 5.18 (0.81) | <0.0001 | 4-10 |
| Neutrophils, x $10^9$/L (SD) | 4.9 (1.5) | 2.9 (0.8) | <0.0001 | 2-7 |
| Total monocytes, x $10^9$/L (IQR) | 0.515 (0.41-0.79) | 0.41 (0.35-0.46) | 0.01 | 0.2-0.8 |
| CRP, mg/L (IQR) | 2.75 (0.77-4.13) | 1.10 (0.625-2.05) | 0.086 | <5 |
| Lowest ABPI, (SD) | 0.73 (0.17) | 1.15 (0.05) | <0.0001 | |

ABPI, ankle brachial pressure index; BMI, body mass index; CRP, C-reactive protein; FMD, flow mediate dilation; HbA1c, glycosylated haemoglobin; HDL, high-density lipoprotein; interquartile range; LDL, low-density lipoprotein; SD, standard deviation; WCC, white cell count.

Table 6.2. The walking performance endpoints following 12-weeks of supervised exercise training in patients who underwent assessment of monocytes (mean ± SD, Student's paired *t*-test).

| **Variable** | **Pre-SEP (n=20)** | **Post-SEP (n=20)** | **Difference, mean (SD)** | **p-value** |
|---|---|---|---|---|
| Pain free walking distance, m | 155 (78) | 185 (96) | 29 (51) | 0.03 |
| Six-minute walking distance, m | 387 (68) | 414 (55) | 27 (38) | 0.005 |

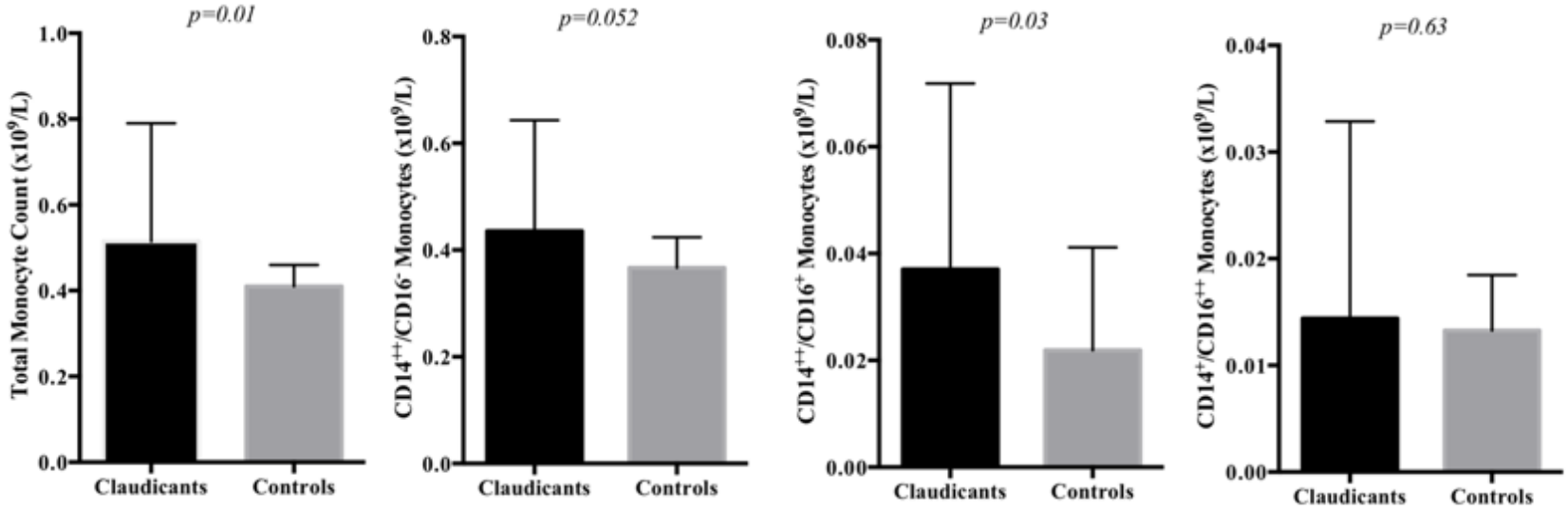


Figure 6.2. Total monocyte and absolute number of monocyte subtypes in patients with claudication and controls. At baseline there was a significant difference in total monocyte count and intermediate monocyte count between patients with claudication and control. There was no significant difference in non-classical monocytes (Mann-Whitney *U* test, bars are median values, error bars are IQR).

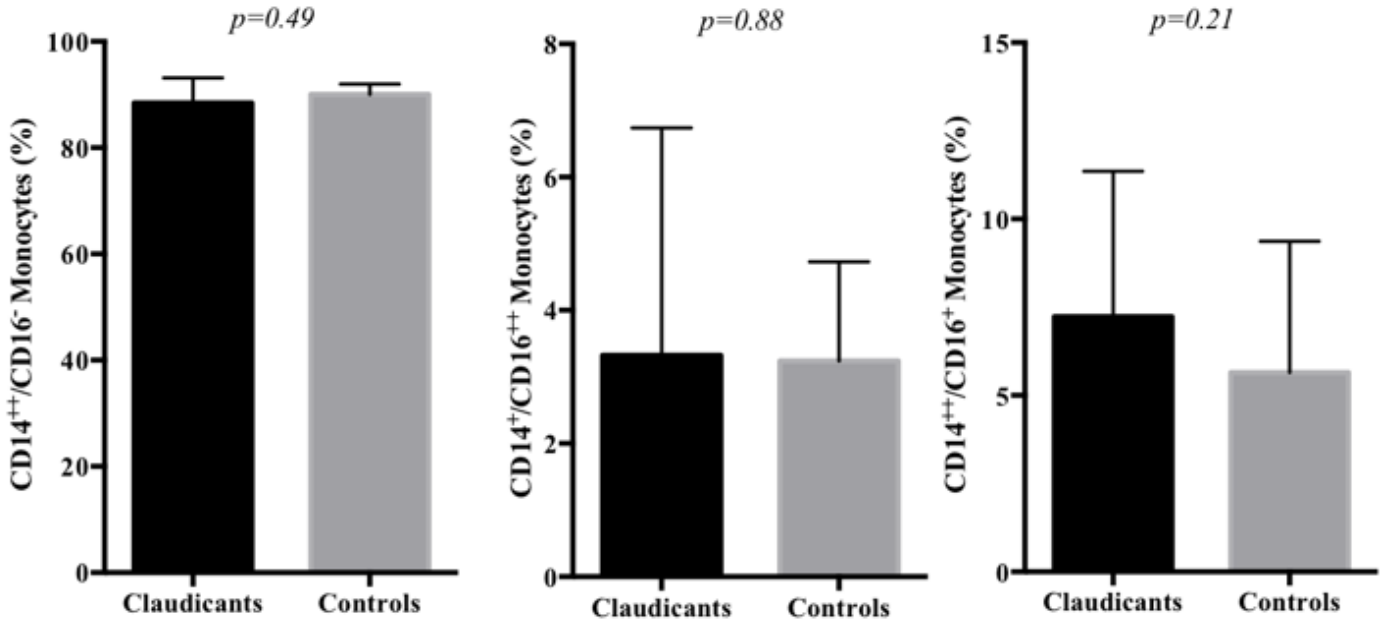


Figure 6.3. Percentage of absolute number of monocyte subtypes in patients with claudication and controls. At baseline there was not significant difference in the percentage of each of the three specified monocyte subtypes (Mann-Whitney *U* test, bars are median values, error bars are IQR).

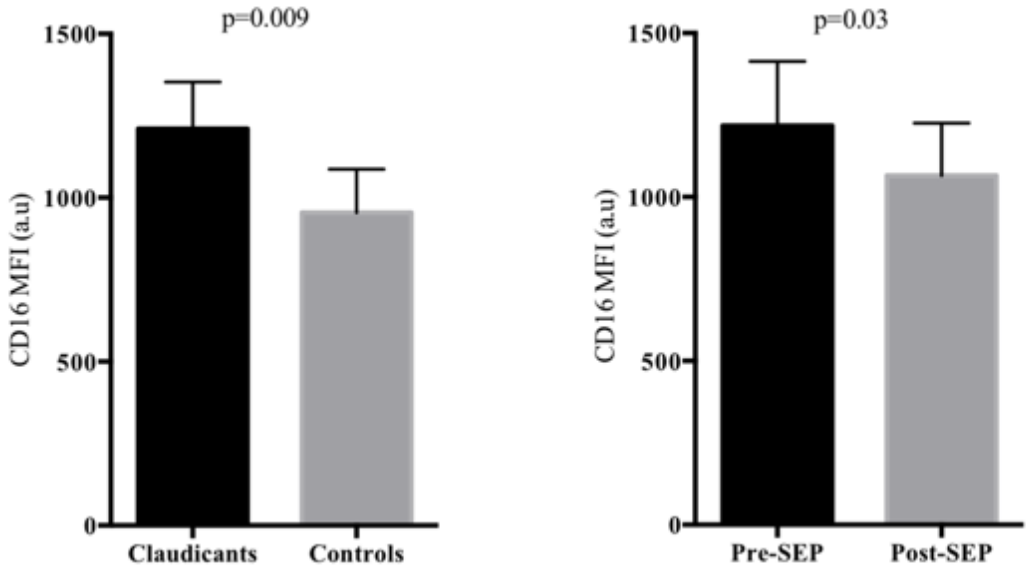


Figure 6.4. Surface expression of CD16 (Fcγ-III receptor) on monocytes in patients with claudication versus controls, and in patients with claudication pre- and post- supervised exercise training. CD16 MFI was significantly higher among patients with claudication compared to controls (Mann-Whitney *U* test). In addition, following completion of the SEP, CD16 expression was significantly lower than at baseline (Wilcoxon matched-pairs signed rank test, bars are median values, error bars are IQR).

Table 6.3. The distribution of monocyte subsets in patients with intermittent claudication at baseline and following 12-weeks of supervised exercise training (Median IQR, Wilcoxon matched-pairs signed rank test).

| **Variable** | **Pre-SEP n=20** | **Post-SEP n=20** | **Difference, median** | **p-value** |
|---|---|---|---|---|
| **Absolute count:** | median (IQR) | median (IQR) | | |
| Classical Monocytes, $x10^9$/L | 0.388 (0.31-0.72) | 0.52 (0.31-0.68) | 0.013 | 0.31 |
| Intermediate Monocytes, $x10^9$/L | 0.037 (0.027-0.07) | 0.038 (0.023-0.072) | 0.001 | 0.98 |
| Non-classical Monocytes, $x10^9$/L | 0.01 (0.005-0.034) | 0.015 (0.004-0.044) | 0.0001 | 0.62 |
| **Percentage:** | | | | |
| Classical Monocytes, % | 88.15 (79.45-93.25) | 87.40 (82.55-92.30) | -0.85 | 0.89 |
| Intermediate Monocytes, % | 8.355 (4.735-12.25) | 8.055 (4.808-12.35) | -0.36 | 0.84 |
| Non-classical Monocytes, % | 3.325 (1.538-6.933) | 3.690 (1.360-5.538) | 0.05 | 0.87 |

Classical $CD14^{++}/CD16^{-}$, Intermediate $CD14^{++}/CD16^{+}$, Non-classical $CD14^{+}/CD16^{++}$.

## 6.5 **Discussion**

Monocytes are key components of the innate immune system and together with macrophages contribute to the development of inflammation and atherosclerosis (Tabas & Bornfeldt, 2016; Tabas & Lichtman, 2017). Monocyte heterogeneity is at concept that was first reported by Passlick *et al.* in 1989 (Passlick, Flieger & Ziegler-Heitbrock, 1989). Functionally, classical monocytes are characterised by an antimicrobial activity, high phagocytic potential, and strong interactions with platelets (Zawada *et al.*, 2011, 2012). In comparison, intermediate monocytes display relatively increased basal ROS and TNF-a production, oxLDL uptake and antigen presentation and stimulation of CD4 T-cell cells (Mosig *et al.*, 2009; Wildgruber *et al.*, 2016b; Cros *et al.*, 2010; Zawada *et al.*, 2011; Rossol *et al.*, 2012). The latter functions are important in atherosclerotic plaque development and progression (Tabas & Lichtman, 2017). Kinetic studies that have suggested that monocytes transition through the subtypes from classical, intermediate to non-classical (Patel *et al.*, 2017). From an epidemiological perspective, it has been demonstrated that a shift toward "inflammatory" intermediate and non-classical monocytes is associated with poorer outcomes in coronary artery disease (Rogacev *et al.*, 2012), chronic kidney disease (Rogacev *et al.*, 2011), and more advanced stages of peripheral artery disease (Dopheide *et al.*, 2012; Wildgruber *et al.*, 2016a). Thus, modification of circulating monocyte subpopulations represents a potential therapeutic target.

Using flow cytometry, Timmerman *et al.* were the first to demonstrate that physical inactivity was associated with a higher percentage of CD16+ monocytes,

that appeared to be lowered following 12-weeks of combined endurance and resistance training (Timmerman *et al.*, 2008). The effect of regular exercise training in patients with intermittent claudication had not previously been investigated, which, was the aim of the present study. Initially, a group of patients with intermittent claudication were compared to a convenience sample of healthy controls. In analysing the monocyte subtype results it is important to note that there appears to be no age dependence, however, healthy females appear to have lower absolute numbers of intermediate monocytes (Heimbeck *et al.*, 2010). Although not matched well for age, gender and a number of other factors (such as medication use and risk factors), patients with claudication appeared to have significantly elevated levels of inflammation given by elevated levels of CRP, WCC, neutrophils and total monocytes. When flow cytometry was used to identify monocyte subpopulations based on consensus definitions, the present study found a significantly higher number of intermediate monocytes in patients with claudication compared to controls. However, there was no significant difference in the numbers of classical and non-classical monocytes between patients with claudication and controls. Furthermore, when monocyte subtypes were analysed as a percentage of the total, there was no significant difference in subtype distribution between patients with claudication and controls. Thus, the observation of higher numbers intermediate monocytes in patients with claudication may simply reflect the higher total number of monocytes. This finding is dissimilar those reported by Dopheide *et al.* 2012 who reported a greater percentage of classical monocytes, and a lower percentage of intermediate and non-classical monocytes, in controls versus patients with PAD (Dopheide *et al.*, 2012). It is noteworthy that in the study by Dopheide *et al*., the control group was well matched for age but not for gender, with 50% of the controls being

female, versus only 23% in the group of patients with claudication. This discrepancy may be due to an inadequate sample size in the present study. Other factors that may plausibly influence monocyte subpopulations are medications such as antiplatelets, statins and antidiabetic medications and controlling for these in the future would be important.

In the present study, patients with intermittent claudication then went on to complete 12-weeks of supervised treadmill exercise and monocyte subtypes were again analysed. No significant change in the defined subtypes was detected between baseline and follow-up in either absolute count or percentage of total monocytes. This was not consistent with a subsequent study by reported by Dopheide *et al.*, in 2015, which demonstrated a significant increase in the percentage of classical monocytes, and significant decrease in intermediate and non-classical monocytes in 40 patients with claudication after 12-months of home-based exercise therapy (Dopheide *et al.*, 2015). This discrepancy between studies may be due to the differences in sample size and duration of the study which was considerably larger and longer than the present study. In addition, the exercise stimulus is likely to be gentler and more gradual when home-based, compared to that of supervised treadmill exercise. Despite these differences, the epidemiological literature suggests that intermediate monocytes are likely to be important players in the progression of atherosclerotic disease, and supporting this notion is the recent evidence high levels are associated with significant restenosis following coronary and peripheral endovascular intervention (Liu *et al.*, 2010; Wildgruber *et al.*, 2016b).

The trichotomy of the monocyte in the abovementioned subsets may, however, be an oversimplification and inherently insensitive (Cappellari *et al.*, 2017). Cappellari *et al.* proposed in their report that monocytes exist in a continuum rather than distinct subsets since higher CD16 expression on monocytes rather than the numbers of specific monocyte subtypes independently predicted adverse cardiovascular outcomes in patients with elevated cardiovascular risk (Cappellari *et al.*, 2017). With this in mind, total monocyte expression of CD16, as given by the mean fluorescence intensity (MFI) of identified monocytes, was measured in the controls and patients with claudication. The present study found that the monocytes of patients with claudication had a significantly higher CD16 expression than controls. In addition, following the completion of the 12-weeks of supervised exercise, monocyte CD16 expression decreased significantly towards the level measured in controls, suggesting that one of the beneficial effects of supervised exercise in patients with claudication is a lowering of monocyte CD16 expression. Lower monocyte CD16 expression may negatively influence the development of atherosclerosis since it has been observed that CD16 expression closely correlated with endothelial adhesive efficiency and circulating inflammatory cytokines (Huang *et al.*, 2012). This observation, combined with the link between intermediate monocytes and restenosis after endovascular intervention described above (Wildgruber *et al.*, 2016b), may possibly explain the synergy resulting in improved walking ability and quality of life observed when supervised exercise therapy is combined with endovascular revascularisation (Fakhry *et al.*, 2018; Mazari *et al.*, 2017).

It is conceivable that regular exercise downregulates the development of intermediate monocytes and CD16 expression via the release of some exercise

induced signalling molecule. Indeed, it has been speculated that exercise causes transient spikes in endogenous cortisol which may have a role in decreasing CD16+ monocytes (Timmerman *et al.*, 2008). This is plausible since intermediate and non-classical monocytes can be depleted by both low and high doses of prednisolone (Dayyani *et al.*, 2003; Heimbeck *et al.*, 2010). Conversely, a chronic exposure to low-dose steroids appears to result in monocytosis and elevated levels of pro-inflammatory intermediate monocytes, at least in kidney transplant recipients (Rogacev *et al.*, 2015). Understanding the effect that supervised exercise has on levels of endogenous corticosteroids in patients with claudication may further our understanding of anti-inflammatory effects of regular exercise. Other signals such as cytokines, miRNAs, vesicles and exosomes may also play a role as hypothesised in recent review articles of "exerkines"(Safdar, Saleem & Tarnopolsky, 2016). Discovery and exploitation of such signalling pathways may have significant therapeutic potential for atherosclerotic and other chronic inflammatory diseases (Yu, Tsai & Kuo, 2017).

## Limitations

There are significant limitations to the present study. Firstly, it is acknowledged that a very small sample size was available in terms of both patients with claudication and controls. With such a heterogenous condition, this decreases the confidence in the endpoint estimations and increases the risk of making type II error. Future studies in this area should seek to define, a priori, the required sample size to minimize the chance of a type II error. Additionally, an adequately powered study would enable the use of multivariable analysis and responder analysis. Networking with other vascular surgical units and combining data multiple centres can increase the pool of potential study participants. Secondly,

the control group was not well matched to patient group and any of the factors that were different between the groups could be responsible for the differences in the endpoints observed. Thus, future studies should follow scientific design principles include appropriate and adequate control groups. Planning for what can is achievable during a short recruitment period is vital to ensure research resources are not wasted. The gold standard would be to perform a prospective randomised controlled trial with patients with claudication randomised to a SEP group, a conservative group, and an exercise and revascularisation combination treatment. Thirdly, the present study looked only at two points in time. A greater understanding of the kinetics of inflammatory monocytes and their effects could be achieved by making measurements at baseline, and following acute exercise and at rest at multiple time points over a longer period of time. Finally, the concept of distinct subsets of monocytes is probably and oversimplification as others have suggested, thus recognizing the alterations in the monocyte continuum maybe more sensitive and accurate model of the underlying pathophysiology.

In conclusion, patients with intermittent claudication have greater numbers of total monocytes and pro-inflammatory intermediate monocytes compared to unmatched healthy controls. In addition, the monocytes of patients with claudication have greater expression of CD16, which appears to decrease following 12-weeks of supervised treadmill exercise training. These findings support the concept of that PAD is characterised by overactive chronic inflammation, and that regular supervised exercise may exert beneficial anti-inflammatory effects on the monocyte pathophysiology.

# 7 ENDOCAN (ESM-1) SERUM LEVELS IN RELATION TO PAD: A CROSS SECTIONAL STUDY

## 7.1 **Abstract**

*Objectives*

Endothelial dysfunction is considered the first step toward atheroma formation and there is much interest in discovery of novel markers of its presence. Endothelial cell-specific molecule-1 (ESM-1), also known as endocan, is a newly discovered marker that can be isolated and measured from the serum of patients. There is currently no data available on the levels of endocan in patients with peripheral artery disease. The primary aim of the present study was to determine the distribution of endocan levels in patients with intermittent claudication and in a convenience sample of healthy non-matched controls. The secondary aim was to determine if endocan levels correlated with other markers of endothelial function: flow mediate dilation (FMD) and reactive hyperaemia index (RHI).

*Methods*

Twenty-seven patients with calf claudication and twenty-one healthy control participants were recruited into the study. Serum endocan levels were assessed using commercially available colorimetric enzyme-linked immunosorbent assay. Flow mediated dilation and RHI were measured using established protocols.

*Results*

There was no significant difference in RHI between patients with claudication and controls ($p=0.44$), however, FMD was approximately double in controls versus patients with claudication ($p<0.001$), and endocan was significantly lower in

controls versus patients with claudication (p=0.04). Serum endocan levels correlated with leukocyte count (r=0.32, p=0.02) and HbA1c (r=0.34, p=0.02), and correlated inversely with FMD (r=-0.31, p=0.04).

*Conclusions*

Serum endocan levels are significantly higher in patients with intermittent claudication compared to healthy controls. Endocan levels were inversely correlated with FMD, and furthermore correlated positively with leukocyte count and HbA1c, suggesting its role as an endothelial inflammatory marker. Endocan may be a less time consuming and operator dependent that brachial FMD.

## 7.2 **Introduction**

As mentioned in section 2.1.6.2, the endothelium plays a central role in the pathogenesis of atherosclerotic diseases. Although it is a cellular monolayer within arteries, in totality, the endothelium is a massive organ that has many important functions in normal vascular homeostasis. Whilst appropriate endothelial function is characterised by maintenance of blood flow though vasodilatory, antithrombotic, anti-inflammatory, and fibrinolytic processes, endothelial dysfunction is defined by inappropriate vasoconstriction and a pro-thrombotic pro-inflammatory state (Gimbrone & García-Cardeña, 2016). In PAD and other atherosclerotic diseases endothelial dysfunction is considered to be the earliest measurable stage of atherosclerosis. The gold standard assessment of endothelial function by way of measuring coronary vasodilation is invasive and costly which has sparked interest in non-invasive tests such as FMD and reactive hyperaemia and blood tests such as NO and nitrate assays. However, these tests are not without their specific technical drawbacks.

Endothelial cell specific molecule-1 (ESM-1), also known as endocan, is a dermatan sulphate proteoglycan that was initially identified as cytokine regulated product of human umbilical vein endothelial cells (Béchard *et al.*, 2000, 2001). Subsequent studies have demonstrated associations with pre-atherosclerotic, atherosclerotic and inflammatory conditions such as hypertension, chronic kidney disease, acute myocardial infarction (Balta *et al.*, 2014; Yilmaz *et al.*, 2014; Qiu *et al.*, 2016). Furthermore, serum endocan levels have correlated with established markers of endothelial function such as FMD, carotid intima-media thickness, and hs-CRP (Balta *et al.*, 2014). No study, however, has investigated endocan levels in patients with peripheral artery disease and claudication. Therefore, the aim of the present study was to determine whether endocan could be used as marker of

endothelial function in patients with PAD and intermittent claudication and its relationship to FMD and RHI. The primary hypothesis was that serum endocan levels would significantly differ between patients with intermittent claudication and healthy controls.

## 7.3 **Methods**

Patients with calf claudication were recruited as described in Section 3.2. Participants acting as non-diseased controls were recruited from the hospital volunteer's organisation and members of staff from the department of vascular surgery. All control participants underwent screening to exclude PAD though history and ABPI testing.

Routine clinical assessment, ABPI determination, and biochemical assessment has been described in Section 3.5.1.

Endothelial function was determined using brachial FMD and peripheral artery tonometry as described in Section 3.5.3.2.1 and 3.5.3.2.2 respectively.

### 7.3.1 **Measurement of Serum Endocan Levels**

Blood for the measurement of endocan was collected into 8mL Vacutainer SST II (Becton Dickinson, Franklin Lakes, New Jersey, USA) serum separator tubes, allowed to clot for 15 minutes, then centrifuged at 3,000g for 10 minutes at 20°C in an Eppendorf 5702 Centrifuge (Eppendorf AG, Hamburg, Germany). Serum was then immediately aliquoted into 2mL RNase and DNase free screw cap microtubes (Thermo Fisher Scientific Australia, Scoresby, Victoria) and frozen at -80°C in a Model 700 series ultra-low temperature freezer (Thermo Fisher Scientific Australia, Scoresby, Victoria) until ready for assay.

Human endocan was measured using a commercially available colorimetric 96-well ELISA kit (MBS705843, MyBioSource, San Diego, CA, USA). The intra-assay coefficient of variation (CV) of this was <8%; the inter-assay CV was <10%. The minimum detectable concentration for endocan was 156 pg/mL. On the morning of assay, 2mL serum aliquots and reagents were thawed to room temperature (18-25°C). Assay procedure was carried out according to the manufacturer's instructions. One hundred microliters of the patient sample was added to the pre-coated 96-well plate and incubated for 2 hours at 37°C in an incubator (Thermo Fisher Scientific Australia, Scoresby, Victoria). The liquid from each well was then tipped out. One hundred microliters of the supplied biotin-conjugated antibody specific for human endocan was then added to the wells and incubated for 1 hour at 37°C. The well contents were then aspirated and the wells washed using 200 μL of the supplied wash buffer allowing it to stand for 2 minutes followed by emptying of well contents. Washing was repeated a further two times. One hundred microliters of the supplied avidin conjugated horseradish peroxidase was then added to the wells and incubated for 1 hour at 37°C. Washing, as above, was then performed five times. Ninety microliters of the supplied tetramethylbenzidine (TMB) chromogenic substrate was then added to the wells and incubated for 15 minutes at 37 °C in darkness. Fifty microliters of the supplied stop solution were then added to the wells. Measurements were then carried out within 5 minutes using an optical ELISA plate reader (Thermo Scientific Multiskan EX microplate photometer (Thermo Fisher Scientific Australia, Scoresby, Victoria) set at 450nm. A standard curve was constructed using a 1:2 dilution series from the supplied known standard and sample diluent. All the samples were assayed in duplicate and averaged.

### 7.3.2 **Measures of Endothelial Function**

Refer to Chapter 3.5.3.2

### 7.3.3 **Statistical analysis**

GraphPad Prism 6.00 for OS X (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Continuous variables were assessed using D'Agostino and Pearson's omnibus normality testing. Variables passing normality testing are expressed as mean ± standard deviation, and those not passing are expressed as median ± interquartile range. Between-group comparisons were made using the student's t test, or Mann-Whitney *U* test when normality not assumed. Categorical variables were compared by Fisher's exact test. Pearson correlation analysis was used to evaluate the relationship between variables of endothelial function. All p-values are reported for two-tailed tests and were assumed significant if $p<0.05$.

#### 7.3.3.1 **Sample Size Justification**

For the primary outcome of serum endocan levels, sample size was calculated based on the assumption of an effect size (Cohen's d) of 1.09 based on results previously reported by Balta *et al*. (mean endocan 1.31 ng/mL (SD 0.72) in hypertensive patients, mean endocan 0.74 ng/mL (SD 0.17) in healthy control patients (Balta *et al.*, 2014). Using G*Power software version 3.1 for OSX (Düsseldorf, Germany), the sample size for a two-sided Mann-Whitney *U* test was calculated *a priori*. Assuming an effect size of 1.09, $\alpha = 0.05$, and $\beta = 0.80$, a total sample size of 30 would be required.

## 7.4 **Results**

Baseline demographic and laboratory data are shown in Table 6.1. The claudicant and control groups were not well matched for either age or gender and these variables were statistically significantly different. In addition, a number of other laboratory values were significantly different between the groups namely homocysteine, triglycerides, total cholesterol, HDL cholesterol, HbA1C, white cell count, and neutrophil count.

Mean endocan levels were significantly higher in in patients with claudication 1.01 ± 0.4 ng/mL versus 0.78 ± 0.36 ng/mL in control participants ($p=0.04$; Figure 6.1). Flow mediated dilation was also significantly different between the groups with the mean FMD 2.9% ± 2.3% in patients with claudication versus 6.3% ± 3.2% ($p<0.001$; Figure 6.2). However, there was no significant difference in the RHI, (patients with claudication and controls: 2.16 ± 0.45 and 2.10 ± 0.49, respectively; $p=0.49$).

In whole study group, there was a significant inverse correlation between age and FMD ($r=-0.43$, $p=0.002$), and between FMD and serum endocan levels ($r=-0.31$, $p= 0.04$) (Figure 6.2), but not between age and endocan or RHI (Table 6.2). There was also a significant inverse correlation between FMD and BMI ($r=-0.35$, $p= 0.02$), FMD and SBP ($r=-0.34$, $p= 0.02$), FMD and HbA1c ($r=-0.34$, $p= 0.04$), FMD and WCC ($r=-0.31$, $p= 0.04$), and FMD and CRP ($r=-0.31$, $p= 0.04$). There was a significant positive correlation between endocan and HbA1c ($r=-0.34$, $p= 0.02$), and WCC ($r=-0.33$, $p= 0.02$) (Table 6.2).

Table 7.1. Demographic features and laboratory findings in patients with claudication and in controls undergoing endocan measurement.

| | Patients with claudication (n=26) | Controls (n=21) | p-value |
|---|---|---|---|
| Age, years (SD) | 66 (11) | 59 (8) | 0.02 |
| Sex (M/F) | 21/6 | 8/13 | <0.01 |
| BMI, kg/m$^2$, mean (SD) | 27.5 (5.3) | 25.9 (2.9) | 0.2 |
| Creatinine, μmol/L, mean (SD) | 78 (19) | 81 (14) | 0.09 |
| Homocysteine, μmol/L, median (IQR) | 13 (10, 16) | 10 (8, 11) | 0.001 |
| Triglycerides, mmol/L, median (IQR) | 1.4 (1.0, 2.2) | 0.8 (0.6, 1.0) | <0.0001 |
| Total cholesterol, mmol/L, mean (SD) | 4.2 (1.4) | 5.1 (1.1) | 0.02 |
| LDL cholesterol, mmol/L, mean (SD) | 2.3 (1.1) | 2.8 (1.1) | 0.11 |
| HDL cholesterol, mmol/L, median (IQR) | 1.3 (1.1, 1.6) | 2.0 (1.6, 2.3) | <0.0001 |
| HbA1c, %, median (IQR) | 5.6 (5.3, 6.6) | 5.3 (4.9, 5.6) | 0.003 |
| Haemoglobin g/dL, median (IQR) | 140 (132, 153) | 138 (132, 148) | 0.34 |
| WCC, x 10$^9$/L, mean (SD) | 7.8 (1.8) | 5.2 (0.8) | <0.0001 |
| Neutrophils, x 10$^9$/L, mean (SD) | 4.9 (1.4) | 2.9 (0.8) | <0.0001 |
| CRP, median (IQR) | 2.4 (0.8, 3.4) | 1.1 (0.6, 2.1) | 0.76 |
| Endocan, ng/mL, mean (SD) | 1.01 (0.4) | 0.78 (0.36) | 0.04 |
| Brachial FMD, %, mean (SD) | 2.9 (2.3) | 6.3 (3.2) | <0.001 |
| Reactive hyperaemia index, mean (SD) | 2.16 (0.45) | 2.1 (0.49) | 0.44 |
| Lowest ABPI, median (IQR) | 0.72 (0.53, 0.83) | 1.15 (1.12, 1.19) | <0.0001 |

ABPI, ankle brachial pressure index; BMI, body mass index; CRP, C-reactive protein; FMD, flow mediate dilation; HbA1c, glycosylated haemoglobin; HDL, high-density lipoprotein; interquartile range; LDL, low-density lipoprotein; SD, standard deviation; WCC, white cell count.

Table 7.2. Correlation analysis of measures of endothelial function and other factors in patients with claudication and controls.

| | FMD (%) | | RHI | | Endocan (ng/mL) | |
|---|---|---|---|---|---|---|
| Variable | *r* | *P-value* | *r* | *P-value* | *r* | *P-value* |
| Age | **-0.43** | **0.002** | 0.20 | 0.17 | -0.19 | 0.20 |
| BMI | **-0.35** | **0.02** | **-0.32** | **0.03** | 0.18 | 0.25 |
| SBP | **-0.34** | **0.02** | -0.01 | 0.90 | -0.08 | 0.55 |
| Creatinine | 0.07 | 0.63 | -0.20 | 0.23 | -0.06 | 0.68 |
| Homocysteine | -0.13 | 0.37 | 0.12 | 0.44 | 0.13 | 0.39 |
| Triglycerides | -0.08 | 0.60 | -0.08 | 0.56 | 0.15 | 0.32 |
| Total Chol | 0.15 | 0.30 | -0.01 | 0.56 | -0.06 | 0.66 |
| LDL-C | 0.11 | 0.46 | -0.60 | 0.71 | -0.06 | 0.70 |
| HDL-C | 0.24 | 0.10 | 0.15 | 0.33 | -0.14 | 0.36 |
| HbA1C | **-0.34** | **0.02** | -0.17 | 0.27 | **0.34** | **0.02** |
| WCC | **-0.29** | **0.05** | 0.02 | 0.87 | **0.33** | **0.02** |
| Neutrophils | **-0.29** | **0.05** | 0.03 | 0.87 | 0.24 | 0.10 |
| CRP | **-0.35** | **0.02** | -0.12 | 0.44 | 0.06 | 0.70 |
| FMD | | | -0.18 | 0.23 | **-0.31** | **0.04** |
| RHI | -0.18 | 0.23 | | | 0.14 | 0.36 |

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FMD, flow-mediated dilatation; HDL-C, high-density lipoprotein; HbA1C, haemoglobin A1C; LDL-C, low-density lipoprotein WCC, white cell count; RHI, reactive hyperaemia index; SBP, systolic blood pressure. Bold values statistically significant $p< 0.05$ was considered significant.

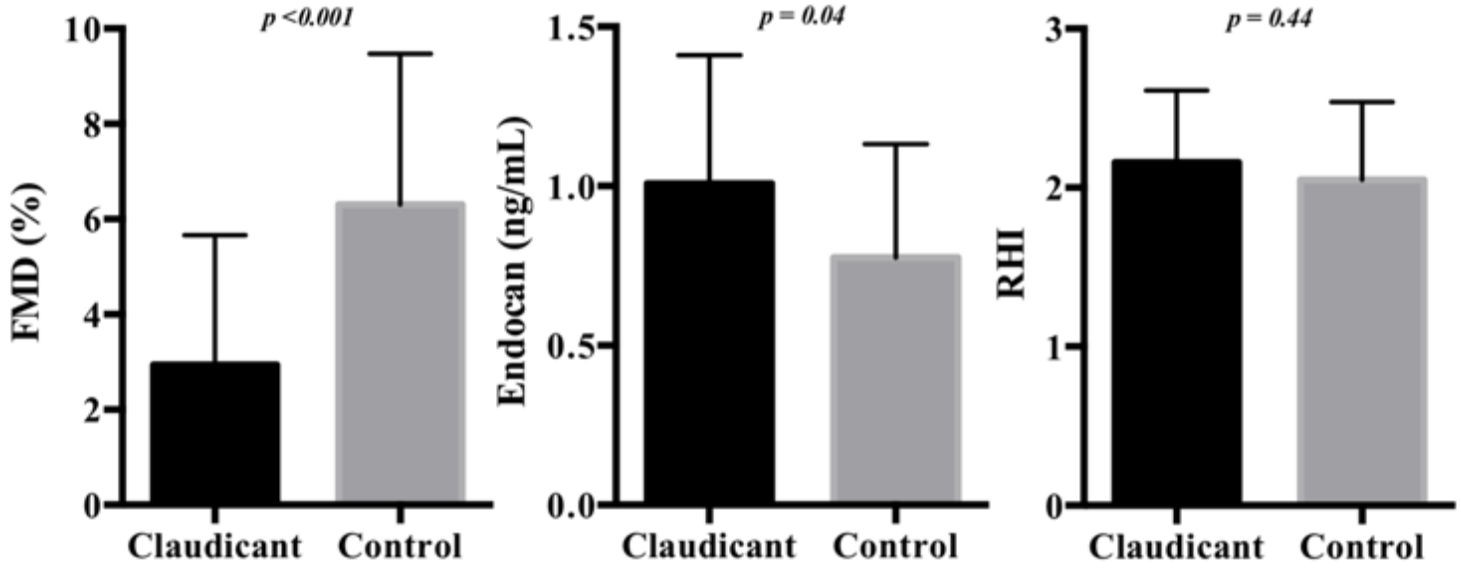


Figure 7.1. Comparison of flow-mediated dilatation, serum endocan, and reactive hyperaemia index in patients with intermittent claudication and controls (Mann-Whitney *U* test, bars are median values, error bars are IQR).

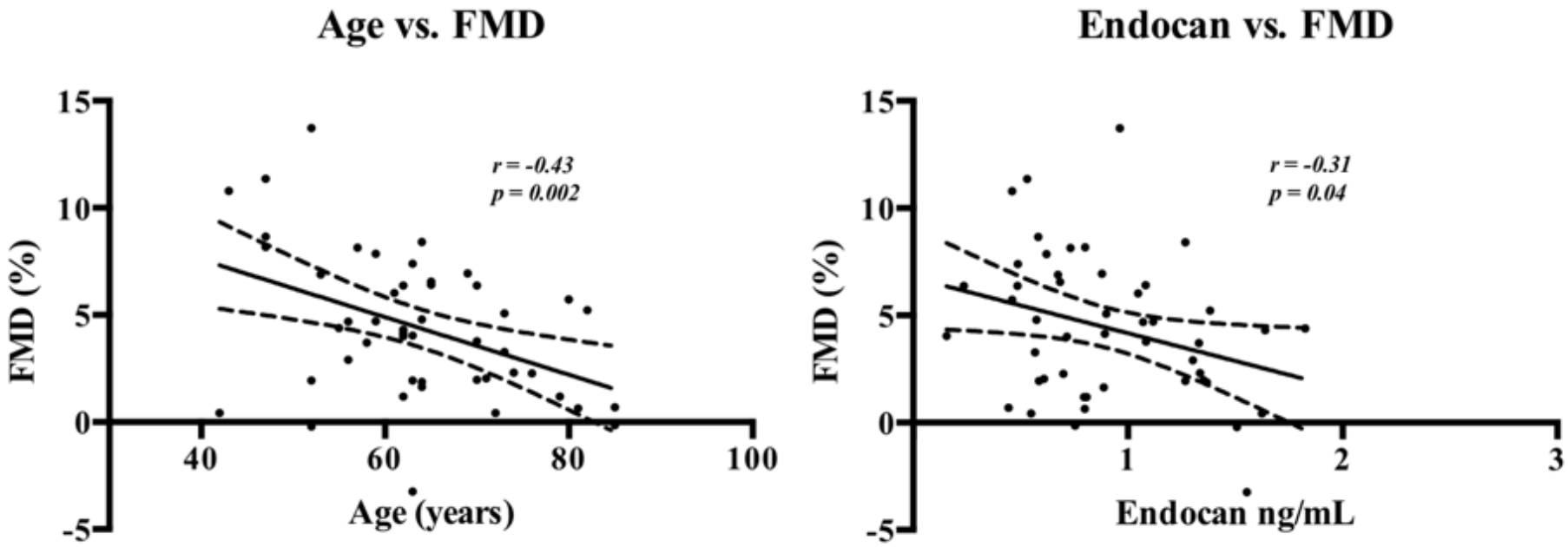


Figure 7.2. The correlation of flow mediated dilatation and age, and endocan (Pearson correlation; dashed lines represent 95% confidence intervals).

## 7.5 **Discussion**

Endothelial dysfunction has been identified as a key early event in the initiation and progression of atherosclerotic disease such as peripheral artery disease. Various markers of endothelial dysfunction have been evaluated in a number of disease states, but no study has investigated the endocan in patients with peripheral artery disease and claudication. The present investigation was a small study with the aim of determining if endocan could be used as a useful marker of endothelial function in patients with claudication, and what factors it is associated with.

This study has demonstrated that endocan is measurable in patients with claudication and levels are significantly higher than in unselected and unmatched control subjects. Importantly, differences in age, gender, risk factors and medication used, homocysteine, triglycerides, total cholesterol, HDL cholesterol, HbA1C, white cell count, and neutrophil count, between patients with claudication and controls probably account for this difference. More significantly, however, an inverse correlation between FMD and endocan was observed and a positive correlation between endocan and known markers of inflammation, WCC and $Hb_A1c$ was also noted. This is consistent with accumulating evidence that endocan levels are elevated in a number of other disease states where endothelial dysfunction and inflammation exist. For example, endocan levels are significantly elevated inpatients with newly diagnosed primary hypertension compared to matched control subjects (Balta *et al.*, 2014). In addition, endocan levels in these patients correlated the inflammatory maker hs-CRP and carotid artery intima-

media thickness, a marker of marker of subclinical atherosclerosis. Endocan levels are also elevated in severe sepsis and predict non-survival (Mihajlovic *et al.*, 2014). More recent work has also confirmed the inverse relationship between serum endocan levels and FMD in patients with the chronic inflammatory disorder sarcoidosis, which is characterised by excessive levels of T-cells and mononuclear phagocytic cells (Aciksari *et al.*, 2018). Endocan levels are also observed to be elevated in type 2 diabetics with subclinical atherosclerosis (Lv *et al.*, 2017). Moreover, endocan levels are reported to correlate negatively with FMD and associate with all-cause mortality and cardiovascular events in patients with non-dialysis dependent chronic kidney disease (Yilmaz *et al.*, 2014). A recent study also demonstrated that serum endocan levels were higher in patients suffering large artery stroke and were able to predicted poorer outcomes (He *et al.*, 2018). Thus, endocan is increasingly associated with poorer outcomes in a diverse range of disease states and it would be reasonable to hypothesise the same would hold true in patients with peripheral artery disease.

Modifiable factors that influence endocan levels may therefore provide a novel therapeutic target for a range of diseases. For example, suppression of endocan expression in prostate and hepatocellular carcinoma models decreases migration and increases apoptosis and autophagy in in vitro studies (Rebollo, Geliebter & Reyes, 2017). Very few human studies have investigated the effect of therapies on endocan levels. One study in patients with obstructive sleep apnoea, an important risk factor for cardiovascular disease, demonstrated a significant reduction in endocan after commencement of continuous positive airway pressure treatment (Altintas *et al.*, 2015). Another study in newly diagnosed hypertensive patients demonstrated significantly decreased levels of serum endocan after 3-months of antihypertensive treatment with amlodipine or valsartan (Celik *et al.*, 2015). Thus,

it appears risk modification treatments may positively impact endocan levels. Apart from large artery stroke there is no data on endocan levels in patients with PAD or claudication. Likewise, there is very little data in regards to the effect of exercise on endocan levels. One group recently reported the effects of endurance training among healthy adults over a period of eight months (Sponder *et al.*, 2017). In this study, 109 subjects performed 150min/week moderate or 75min/week vigorous exercise which resulted in a significant exercise test performance increase that was associated with progressively increasing serum levels of endocan. The observation that endocan levels increased with physical performance might suggest that endocan likely plays important roles in both beneficial physiological adaptations and pathological processes. From mechanistic point of view, proangiogenic factors such as VEGF, may provide the link between exercise and elevated levels of endocan since exercise has been demonstrated to increase serum and muscle levels of VEGF, and that VEGF-A is a specific inducer of endocan transcription and translation in human endothelial cells (Rennel *et al.*, 2007).

This study has some important limitations. Firstly, this small study was conducted with a control group that was not well matched for age or gender, thus it is possible that the differences observed were due to factors other than the presence or absence of PAD. In future studies, specific attention to the selection of appropriately matched controls will allow more meaningful comparisons as this is part of good study design universally. Additionally, an investigation of endocan levels in other patients along the spectrum of PAD, such as those with asymptomatic disease and those with critical limb threatening ischaemia will provide greater insights into its pathophysiological role. Secondly, this cross-sectional study cannot establish a cause-effect and provides no information of the

ability of endocan levels to indicate the risk of future cardiovascular events, nor the effect of interventions in claudication. Again, future studies should be designed to help clarify these issues. Thirdly, the small sample size of this study prevents a meaningful multivariate analysis of factors. With multiple comparisons, appropriate adjustments to acceptable significance levels will be required, for example with a Bonferroni correction. Future, studies of endocan in PAD can incorporate this early data to sample size calculations to ensure adequate statistical power.

In conclusion, serum endocan levels are measurable in patients with PAD and claudication and are significantly elevated in comparison to unmatched healthy controls. Furthermore, serum endocan levels correlate negatively with FMD, and correlated positively with worse glycaemic control and total WCC. Further studies are needed to clarify the relationship between the severity of PAD and endocan levels, and what effect exercise and revascularisation might be.

# 8 GENERAL DISCUSSION AND CONCLUSION

## 8.1 **Summary**

Peripheral artery disease and intermittent claudication continue to be significant burdens to patients and the healthcare system. Improving symptoms, function and quality of life is a key goal for health professionals who treat patients who suffer with intermittent claudication. Chapter 2 has highlighted that present treatment strategy is to manage risk factors medically to improve longevity, and recommend regular exercise to improve symptoms. Supervised exercise is now well established as an evidence-based treatment to improve walking performance a majority of patients. This is reflected in the recent decision by Centres for Medicare and Medicaid Services decision to fund supervised exercise programs in USA (Jewell, Shishehbor & Walsworth, 2017). Despite a strong level of evidence that exercise works, how and why this occurs has been the focus of considerable debate and investigation over the last 50 years or so, with a significant focus on muscle, endothelial and immune function. The central aim of this thesis was to investigate further the effects of supervised treadmill exercise on these functions.

In Chapter 4, the effect of treadmill-based exercise on lower limb muscle mass was investigated further since it had previously been hypothesised to have an adverse effect, possibly though repeated exposure to ischaemia-reperfusion. Unlike previous work, this study segmented the data into proximal and distal lower limb segments. It the current thesis, it was hypothesised that atrophy of the calf muscles would account for the previously observed decreased in lower limb muscle mass that followed supervised treadmill exercise, since they are the symptomatic ischaemic end organ in this situation. Surprisingly, however, there

was no significant change in the muscle mass of the calf in either asymptomatic or symptomatic legs following 12-weeks of treadmill training. Even more surprising was the finding that there was a significant reduction in thigh muscle mass following the training. Whether this represents an adverse effect of supervised exercise on muscle is not yet known. Patients improved their overground walking ability despite this change, and thus it might represent a functional adaptation. Conversely, this change may have occurred as a result of some circulating factor invoked by the repetitive exposure to ischaemia. Alternatively, it may reflect the unique biomechanical environment of the treadmill, which is known to result in greater activation of proximal gluteal musculature compared to overground walking. This may be a may be a non-hypertrophic stimulus due it its repetitive, low resistance nature. Whilst the significance of these findings remains unknown, it can be concluded with quite high confidence that supervised treadmill exercise does not lead to atrophy of the calf muscles in the setting of calf claudication. How and why supervised treadmill exercise leads to a reduction in thigh muscle mass remains unanswered and should be a focus of future studies in this area. It has already been shown that patients with claudication can add muscle mass when resistance-training is combined with walking training. Measuring lower limb muscle mass in patients undergoing overground supervised training, compared to treadmill training, may reveal a biomechanical mechanism. Alternatively, measuring regional lower limb muscle mass in patients undergoing revascularisation in combination with exercise training, versus exercise training alone, may reveal a role played by ischaemia.

In Chapter 5, the effect of 12-weeks of supervised exercise on endothelial function and shear-stress related microRNA was investigated. The exercise

intervention failed to improve endothelial function as measured by EndoPAT or FMD. The 12-weeks of supervised treadmill exercise, however, appeared to downregulate miR-92a in both skeletal muscle and serum. Since miR-92a is regulated by shear-stress and is considered proatherogenic and harmful in the setting of ischaemia-reperfusion injury, this exercise induce response probably reflects a beneficial effect to exercise. The measurement of miR-92a in response to exercising patients with PAD had not be performed before and may prove to be a more sensitive marker of endothelial function than FMD or PAT. Further studies are needed to establish the sensitivity of miR-92a in comparison to a reference standard of endothelial function in a large sample. In addition, longitudinal studies incorporating miR-92a as marker will inform whether it predicts cardiovascular complications.

In Chapter 6, the effect of PAD and exercise on circulating monocyte subtypes was investigated. Monocytes play a key role in the pathogenesis of atherosclerosis and the effect of exercise on monocyte levels in this disease group had not previously been investigated at the time of the study. Firstly, patients with PAD were found to have elevated levels of inflammatory cells both neutrophils and monocytes compared to sample of healthy unmatched controls. In addition, the level of 'intermediate' monocytes, considered to be pro-inflammatory and pro-atherogenic, was greater in patients with PAD. It was hypothesised that exercise might decrease the levels of inflammatory subtypes of monocytes, thus suggesting beneficial effect of exercise in patients with claudication. No measurable change in the proportions of monocyte subtypes was detected. However, recent work has challenged the concept of three distinct subgroups of monocytes as an oversimplification. In this study, therefore, the general monocyte population was

reassessed for expression of CD16. In doing this, it was found that monocyte CD16 expression decreased following completion of supervised treadmill training. This may represent a beneficial immune effect of exercise therapy given experimental evidence on the role CD16 plays in vascular adhesion and the development of atherosclerosis (Huang *et al.*, 2012).

Finally in Chapter 7, a novel marker of endothelial activation, endocan, was measured in patients with intermittent claudication and compared to alternative tools for the assessment of endothelial function, FMD and PAT. Whilst the study is not adequately designed to compare endocan between patients with claudication and healthy controls (due to being unmatched), interesting positive correlations were noted between endocan and age, total leukocyte count, HbA1C, along with a negative correlation with FMD. Thus, endocan may be a useful marker of endothelial activation in patients with PAD.

## 8.2 **Limitations**

The short-term nature of the studies in this thesis must first be acknowledged, however, this is similar to previous studies which tend not to be conducted with long-range follow-up. This limitation affects all studies in this thesis, but would be the easiest limitation to overcome in future work should the motivation exist to continue to seek long-term data.

In regard to Chapter 4, the most significant limitation was lack of a control group of either an attention control (no exercise) group or an endovascular revascularisation group. This could be justified because technological advancements that have occurred over the past 5-10 years allow more aggressive

treatment of claudication by endovascular means with improved durability. Additionally, long-term data from RCTs combining exercise therapy with revascularisation has shown synergistic effects compared to either modality alone (Klaphake *et al.*, 2018). Therefore, an optimal future study would combine at least one control group, or alternative treatment group such as modern endovascular treatment, in which the participants have been randomised. However, more treatment arms require a greater sample size which may be a barrier to single centre research. Collaboration with other vascular units is likely needed to overcome this. Secondly, supervised training is not feasible for all patients, thus many potential participants were excluded from the present studies due to their inability to fit in to the hospital schedule. In this regard, developing a protocol of remote supervision in a community-based setting is needed. In addition, the focus in Chapter 4 was primarily the measurement of skeletal muscle mass. At present, it is unknown if changes in skeletal muscle mass translate to positive functional changes and changes in quality of life. Future studies should include measures of strength, function and quality of life.

In regard to Chapter 5, this was a novel endeavour focused on understanding how miR-92a levels change as a result of supervised exercise. As already stated above, the lack of a control group is a significant limitation, however, this was designed to maximise the sample size available for within-group comparison. Indeed, despite this approach, this study was still underpowered based on the *a priori* sample size calculation. Nevertheless, the work presented serves as useful preliminary data which will influence the design of future studies in this space. Additionally, a measure of the downstream effectors of miR-92a was not able to be performed due to time and resource constraints. It would be important to

confirm that a decrease in miR-92a levels is accompanied by increases in the expression transcription factors KLF2 and 4, eNOS, and decrease in the expression of the pro-inflammatory transcription factor NF-κB. Of course, when increasing the number of variables in an experiment, one must also increase both the sample size and correct for multiple comparisons by applying more stringent significance thresholds, such as by the Bonferroni correction method.

Similarly, Chapter 6 also was affected by the limitations of sample size, lack of control group, multiple comparisons, and short duration of follow-up. In addition, only two time points were measured during the course of the study. Therefore, the acute and chronic dynamics of inflammatory elements of the immune system remain unknowable by this design. Increasing the data collection to assess the acute response to exercise, and its chronic adaptations would be an important component of future study design.

In Chapter 7, the study sought to ascertain whether endocan was a useful marker of endothelial function in patients with claudication. The most important limitation of this study was the control group available was not matched for many characteristics. Indeed, the age, gender profile and presence of risk factors differed significantly such that their effect on endocan levels could not be excluded. A future study with less stringent time and resource constraints could be designed to include appropriately matched health controls as well as those with more advanced disease such as CLTI. As already mentioned, multiple comparisons would need to be addressed by increasing the sample size and applying multiple comparison correction procedures such as the Bonferroni correction.

## 8.3 **Conclusion and Implications for Practice and Research**

In conclusion, the results of this thesis suggest that supervised exercise may have a harmful effect on proximal muscle form and function. How this effect clinical translates can only be speculated at this stage. Loss of proximal muscle mass can be hypothesised to have negative effect on mobility and survival, given the impact of sarcopaenia on functional long-term outcomes. Harmful effects on endothelial function or immune function were not observed within the limitations of the presented studies.

With the increasing evidence that structured and supported home-based exercise is as effective as supervised exercise at improving, it would be important assess whether this type of training has differing effects on muscle, endothelial and immune function in future studies. Future research will need to also need to consider the long-term term effects of training on walking ability, but attention also needs to be paid to other activities of daily living that may be impacted by changes in muscle mass such as balance, sitting to standing transfers, and prevention and recovery from falls. In addition, a focus on the long-term cardiovascular outcomes and survival cannot be neglected when attempting to definitively determine the optimal treatment strategy to reduce pain and improve walking ability in patients with intermittent claudication.

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# APPENDIX A – TIMETABLE OF SUPERVISED EXERCISE PROGRAM AND EDUCATIONAL SESSIONS

**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 COHORT 3A**
**Please report to Reception A in the Rehabilitation Building on arrival.**

Following the 1- hour gym session there is a series of short lectures on Peripheral Artery Disease (PAD). The Monday education lectures are conducted in Group Room 2 in the new **Rehabilitation Building at Repatriation General Hospital**. They are open to anyone, including your family, 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. The Thursday physiotherapy sessions are for 1 hour in the gymnasium.

| | Date | Venue | Speaker | Topic |
|---|---|---|---|---|
| **Week 1** | Monday 10th February 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Vascular. CNC | Introduction to program+ Arterial Disease-risk factors |
| | Thursday 13th February 10.30am-11.30am | Physio Gym | | |
| **Week 2** | Monday 17th February 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Physiotherapist | The benefits of a walking program |
| | Thursday 20th February 10.30am-11.30am | Physio Gym | | |
| **Week 3** | Monday 24th February 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Pharmacist | Medication & arterial disease |
| | Thursday 27th February 10.30am-11.30am | Physio Gym | | |
| **Week 4** | Monday 3rd March 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Occupational Therapy | Life style & goal setting |
| | Thursday 6th March 10.30am-11.30am | Physio Gym | | |
| **Week 5** | Monday 10th March | **PUBLIC HOLIDAY** | | |
| | Thursday 13th March 10.30am-11.30am | Physio Gym | | |
| **Week 6** | Monday 17th March 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Dietician | Diet specific for arterial disease |
| | Thursday 20th March 10.30am-11.30am | Physio Gym | | |

| | Date | Venue | Speaker | Topic |
|---|---|---|---|---|
| **Week 7** | Monday 24th March 10.30am-11.30am | Physio Gym | | |
| | 11.45am-12.15pm | (Group Room 2) | Podiatrist | The importance of healthy feet |
| | Thursday 27th March 10.30am-11.30am | Physio Gym | | |
| **Week 8** | Monday 31st March 10.30am-11.30am | Physio Gym | | |
| | Thursday 3rd April 10.30am-11.30am | Physio Gym | | |
| **Week 9** | Monday 7th April 10.30am-11.30am | Physio Gym | | |
| | Thursday 10th April 10.30am-11.30am | Physio Gym | | |
| **Week 10** | Monday 14th April 10.30am-11.30am | Physio Gym | | |
| | Thursday 17th April (10.30am-11.30am) | Physio Gym | | |
| **Week 11** | Monday 21st April 10.30am-11.30am | **PUBLIC HOLIDAY** | | |
| | Thurs 24th April 10.30am-11.30am | Physio Gym | | |
| **Week 12** | Mon 28th April 10.30am-11.30am | Physio Gym | | |
| | Thurs 1st May 10.30am-11.30am | Physio Gym | | |

# APPENDIX B - PROTOCOL FOR ULTRASOUND GUIDED PERCUTANEOUS SKELETAL MUSCLE BIOPSY

Equipment Required

- Ultrasound machine
- Marking pen
- Examination table
- x 2 disposable fenestrated drapes
- 5g EMLA cream and x2 small occlusive dressings (Opsite or IV3000)
- Chlorhexidine prep or equivalent
- Dressing pack and disposable forceps
- 10mL syringe
- Drawing up needle, x 2 22 g needles
- 10-15mL of 1% lignocaine solution for injection
- x2 15G BARD TRUGuide 7.8cm trocars with stop guides
- x1 16G BARD MAXCore 16G 10cm biopsy instrument
- Sterile gloves
- Sterile gauze
- Cohesive bandage
- Normal saline for washing blood
- Collection containers and media (eg. 1-2mL Cyrotubes, RNA*later*®, Buffered Formalin 10%, liquid nitrogen)

1. With the patient supine on the examination table, the symptomatic leg is marked with ultrasound.

a. The thigh is marked by identifying a region of fleshy muscle belly with free of large blood vessels. The vastus lateralis muscle is the preferred muscle; however the vastus medialis can also be used.
b. Depth to fascia and appropriate needle angle and safe depth are noted.
c. The skin is marked with indelible ink felt pen and EMLA is applied with the standard dressing.
d. The procedure is repeated again for the medial calf muscle.

2. After 20-30min with EMLA applied, the patient returns to the examination table in the same position the he was for the marking and the EMLA and dressings are removed. The ink marking may need to be redrawn.
3. Using sterile technique, the patient is prepped with chlorhexidine solution and then draped with a disposable fenestrated drape.
4. 1% lignocaine is then infiltrated along the intended biopsy tract in the thigh and the calf and time given for effect to occur.
5. A 15G 7.8cm BARD TRUguide trocar is inserted along the planned biopsy tract and a depth stop guide prevents unintentional malposition.
6. The 16G 10cm BARD MAXCore biopsy instrument is cocked.
7. Through the TRUGuide trocar, the MAXCore biopsy device is inserted to the full depth. On activation of the biopsy device, the tip of the needle protrudes to a depth of 1.8cm beyond the trocar resulting in the collection of a core of muscle tissue.
8. The biopsy instrument is re-cocked revealing the tissue sample, which is removed with a pair of clear forceps or a clean 22G needle.
9. Further passes with the biopsy instrument are made 4-5 times through the existing trocar and collected tissue is divided into required storage media.

10. When sampling is complete, the trocar is removed and the firm pressure is applied for 5min with clean gauze.
11. The same procedure is repeated for the other region of the leg to be biopsied.
12. When haemostasis is observed, the biopsy sites are dressed with fresh gauze wrapped in a cohesive bandage and an ice pack applied.
13. Advice is given to the patient regarding expected pain, rest and analgesia. Panadol and if necessary, Panadeine forte or alternative moderate analgesic is recommended.
14. All patients are returned to the care of a responsible adult.
15. A follow-up phone call is made 6-8 hours following the biopsy to ensure welfare and contact details and advice of what to do in the event of complications are given.

# APPENDIX C - REAGENTS AND EQUIPMENT USED FOR miRNA QUANTIFICATION.

| | | |
|---|---|---|
| Chemicals and Reagents | | |
| Chloroform<br>Ethanol<br>Isopropanol | | Chem-supply, Gillman, SA, Australia |
| RNA*later*® | | Ambion, Foster City, CA, USA |
| TaqMan® Gene Expression Master Mix<br>TaqMan® MicroRNA Reverse Transcription Kit<br>TaqMan® Universal PCR Master Mix No AmpErase® UNG, | | Applied Biosystems, Foster City, CA, USA |
| TRIzol® Reagent<br>TRIzol-LS® Reagent<br>UltraPure™ Glycogen | | Invitrogen, Newcastle, NSW, Australia |
| Equipment | | |
| Allegra X-22 R centrifuge | | Beckman Coulter, Brea, CA, USA |
| Eppendorf micro centrifuge 5424 | | Eppendorf, Hamburg Germany |
| GeneAmp PCR System 9700 thermal cycler | | Applied Biosystems, Foster City, CA, USA |
| Nanodrop-8000 spectrophotometer | | Nanodrop Technologies, Wilmington, DE, USA |
| Rotor-Gene Q | | Qiagen, Valencia, CA, USA |
| TaqMan® assays | Catalogue number | |
| hsa-miR-92a<br>RNU6B<br>cel-miR-54 | #000430<br>#001093<br>#001361 | Applied Biosystems, Foster City, CA, USA |

# APPENDIX D – PUBLISHED MANUSCRIPTS

## The effect of supervised exercise therapy for intermittent claudication on lower limb lean mass

Simon Vui Vun, BMedSc, MBBS (Hons),[a] Michelle D. Miller, MNutDiet, PhD,[b] Christopher L. Delaney, BM, BS, PhD,[a] Richard B. Allan, BHlthSc (Hons), DMU,[a] *and* J. Ian Spark, MBChB, MD,[a] *Bedford Park, South Australia, Australia*

**ABSTRACT**

**Objective:** Supervised exercise is currently recommended for the first-line treatment of intermittent claudication based on improvement in walking capacity. However, the promotion of skeletal muscle atrophy by repetitive ischemia-reperfusion caused by treadmill-based programs remains a concern. Because preservation of skeletal muscle mass (SMM) and lean mass (LM) is integral to functional capacity and longevity, this study measured the effect of standard treadmill-based supervised exercise on SMM and regional lower limb LM in patients with intermittent claudication.

**Methods:** Patients with calf claudication caused by infrainguinal peripheral artery disease underwent whole-body dual-energy X-ray absorptiometry scanning before and after completion of a 12-week supervised treadmill exercise program. Total body SMM and lower limb LM were measured according to anatomical regions of the lower limb (thigh vs calf) and side of symptoms. Walking performance was assessed using pain-free walking distance and 6-minute walking distance tests.

**Results:** Thirty-six patients with calf claudication completed exercise training and dual-energy X-ray absorptiometry scanning, allowing analysis of 55 symptomatic and 17 asymptomatic lower limbs. No difference in total body SMM ($P = .41$) or LM of symptomatic ($P = .53$) or asymptomatic calves ($P = .59$) was detected after the program. In contrast, a significant decrease in LM was observed in symptomatic ($P = .04$) and asymptomatic thighs ($P = .005$). Pain-free walking distance ($P = .001$) and the 6-minute walking distance both improved significantly ($P = .004$) but were not associated with changes in LM.

**Conclusions:** Twelve weeks of standard treadmill-training for intermittent calf claudication did not result in loss of calf LM; however, a significant decrease in bilateral thigh LM was observed, even in patients with unilateral symptoms. Further research on optimum exercise modalities and end points are required to determine the pathophysiology and effects of these changes on function and survival. (J Vasc Surg 2016;64:1763-9.)

Peripheral artery disease (PAD) is an atherosclerotic disease estimated to affect >200 million people globally.[1] Ischemic muscle pain affecting the legs is the most common manifestation of PAD, and many guidelines prescribe supervised walking exercise as the first line of treatment based on growing evidence of short-term improvements in walking performance.[2-5] In contrast to interventions for other cardiovascular diseases, evidence is sparse regarding the mechanisms that improve walking performance or whether they translate into survival benefit.[6] Alterations to muscle function are thought to explain some of the improvement in walking performance as a result of supervised exercise programs (SEPs), with a number of studies demonstrating changes in muscle architecture, mitochondrial content, and fiber type.[7,8] These adaptations are currently thought to be beneficial; however, repetitive exercise of an ischemic muscle group is accompanied by an ischemia-reperfusion phenomenon that may be deleterious to muscle function and, perhaps, the whole patient longer-term.

Previous work has demonstrated that patients with increasing severity of lower limb PAD have reduced calf muscle cross-sectional area suggesting a negative relationship between ischemia and muscle mass.[9] In addition, lower limb muscle strength is adversely affected by chronic ischemia, with lower ankle-brachial pressure index (ABI) values associated with weaker lower limb strength.[10,11] However, treadmill-based exercise programs have not been found to improve lower limb strength.[12,13] In fact, a loss of bilateral leg strength has been reported when patients change from a resistance-training program to a treadmill-training program.[13]

Given the known association between lower limb strength and death in patients with PAD, preservation of strength and muscle mass is undoubtedly vital.[14]

From the Department of Vascular Surgery,[a] and Nutrition and Dietetics,[b] Flinders University.

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Correspondence: J. Ian Spark, MBChB, MD, Department of Vascular Surgery, Flinders University, Flinders Dr, Bedford Park, SA 5007, Australia (e-mail: ian.spark@sa.gov.au).

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Moreover, a recent randomized controlled trial in which standard treadmill walking was compared with combination walking and resistance exercise demonstrated that a treadmill-only exercise program elevated activity of muscle calpain, a protease implicated in myocyte damage and apoptosis, whereas combination training did not.[15] In addition, change in lean mass (LM) after 12 weeks of treadmill-only training showed a decline, whereas symptomatic lower limb LM increased when combined with resistance-training.[15] Together this suggests that prolonged exercise of muscle groups under ischemic conditions may accelerate muscle atrophy.

What is not known is whether the changes in muscle mass are regional, affecting proximal or distal muscle groups related to blood supply. This study therefore tested the hypothesis that standard treadmill training for calf claudication adversely affects regional lower limb skeletal muscle mass (SMM), despite improvements walking performance, in patients with calf claudication secondary to infrainguinal PAD.[16]

## METHODS

The Southern Adelaide Local Health Network Human Research Ethics Committee approved this study, and participants provided written informed consent.

**Sample.** Patients presenting to the Southern Adelaide Local Health Network Vascular Surgery outpatients clinics between January 2013 and December 2014 were screened for eligibility. Inclusion criteria consisted of a clinical history of calf claudication along with low ABI (<0.9) and duplex ultrasound scanning or computed tomography angiographic evidence of infrainguinal disease in the absence of significant aortoiliac disease. Patients were excluded if they (1) had evidence of rest pain or tissue loss; (2) had recently (<12 months) undergone peripheral vascular revascularization; (3) had recent exercise training; (4) suffered blood dyscrasias or were anticoagulated; or (5) had cardiorespiratory morbidities limiting exercise capacity. Those who met the inclusion criteria were offered a 12-week program of twice-weekly supervised treadmill walking.

Additional data on 11 patients who were part of a treadmill-only training arm of a randomized controlled trial were also included in the data analysis.[17] Inclusion and exclusion criteria were identical between studies, as were the exercise regimen, as described below, and the measurements of physical variables and collection of blood and tissue samples.

**Exercise regimen.** The SEP lasted 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 a senior clinical physiotherapist or exercise physiologist with experience in administering exercise interventions for patients with cardiorespiratory disease. In accordance with international guidelines, participants were instructed to begin walking at a speed and incline to induce claudication pain within 3 to 5 minutes, stop and rest until pain abated, then repeat for the duration of the 1-hour session.[3] Initial treadmill speed was commenced at the average speed calculated from the result of the individual's initial 6-minute walk distance (6MWD) test. During each training session, the physiotherapist monitored the patient's progress to ensure onset of symptoms ≤5 minutes of walking. If patients were able to walk >5 minutes without symptoms, the workload of the treadmill was first adjusted by increasing the speed up to the patient's maximum safe walking speed and then by increasing the incline.

**Exercise testing.** All participants were assessed by a physiotherapist within the week before commencing the exercise program and within 1 week after completing the program using a standardized over ground 6MWD test.[17] The pain-free walking distance (PFWD), defined as the distance at the first sign of claudication, and the total 6MWD were recorded.

**Testing.** Between 1 and 2 weeks before and after the 12-week exercise program, participants attended a research clinic where comorbidities and current medications were recorded and routine blood investigations, body measurements, and dual-energy X-ray absorptiometry scans (DEXAs) were obtained, as described below.

**Regional muscle mass quantification.** Whole-body composition scanning was performed using a DEXA system (Lunar Prodigy, GE Healthcare, Hertfordshire, United Kingdom) with EnCORE 10.51.006 software (GE Healthcare). Participants were positioned supine on the DEXA table top with their feet in a neutral position and hands flat by their sides. After acquisition, whole-body composition was estimated using default automated segmentation. Appendicular soft tissue LM was then used to determine total body SMM according to previously validated methods.[18]

For regional body composition, custom regions of interest (ROI) were traced manually over the DEXA planogram to segment the lower limb into thigh and leg regions. This study defined the thigh as the part of the lower limb between the hip and knee joint and the calf as the part of the lower limb below the knee joint. ROI borders were adapted from previously validated methods.[19]

The proximal extent of the thigh ROI was modified because it was defined by an angled line connecting the lateral aspect of the anterior superior iliac spine and the inferior ramus of the pubis. This line is influenced by sex, with men having a more vertical line than women, given their taller and narrower pelvic structure. The potential effect of this is exclusion of proximal medial thigh musculature and contamination by lower

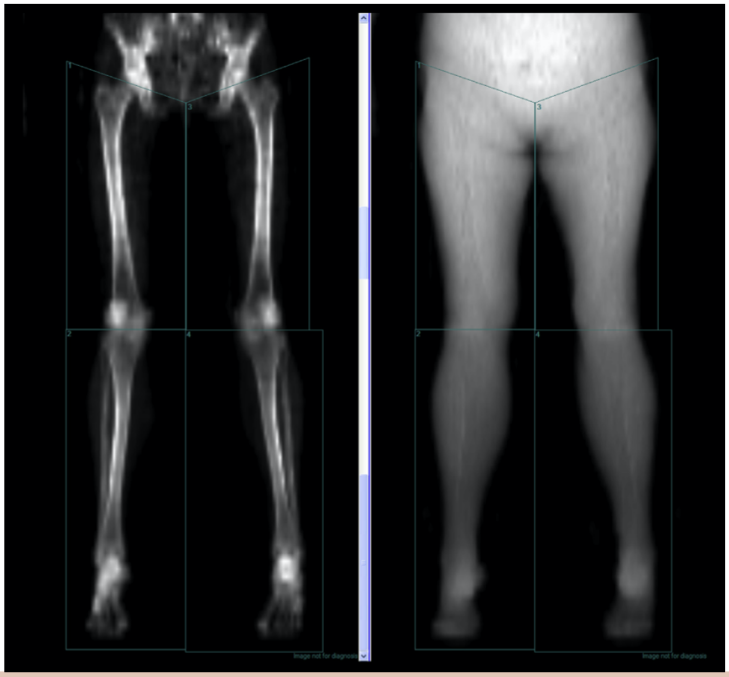

**Fig.** Custom regions of interest (ROI; *green lines*) traced over constant bony landmarks for segmentation and regional analysis of lean mass (LM).

abdominal mass, particularly in obese individuals. Thus, the thigh ROI used in this study was defined proximally by an angled line drawn from the center of the pubic symphysis to the superior aspect of the greater trochanter, distally by a horizontal line through the center of the knee joint space, and medially and laterally to include all soft tissue. The calf ROI was defined proximally by a horizontal line through the center of the knee joint space and distally to include the foot and toes; medially and laterally, all soft tissue was included. The Fig demonstrates the DEXA segmentation for one patient.

**Validation of manual segmentation.** To validate this method of manual segmentation, two assessors used the above definitions to independently segment DEXA scans from 15 randomly selected patients. One assessor repeated the measurements >1 week later. Measurements of LM were recorded, and the Pearson correlation coefficient (*r*), mean coefficient of variation, and intraclass correlation coefficients (ICCs) were used to quantify between and within-assessor reliability.[20] Good to excellent reliability was accepted if ICC was ≥0.75.[21]

**Statistical analysis.** Results for continuous variables are expressed as means and standard deviation (SD). The Student paired *t*-test was used for within-group comparisons and independent samples *t*-test was used for between group comparisons. Because symptoms were unilateral in some patients and bilateral in others, data from each leg were considered as separate entities. The Pearson correlation was used to assess the association among changes in PFWD, 6MWD, SMM, and LM across limb region and symptom status. For correlations, the limb with the lowest ABI was used when symptoms were bilateral. All tests were performed two-sided, and $P < .05$ was considered to indicate statistical significance. GraphPad Prism 6.00 software (GraphPad Software, La Jolla, Calif) was used for statistical analyses.

## RESULTS

Between February 2013 and December 2014, 98 patients were assessed for eligibility into the study. Excluded were 16 patients with no evidence PAD causing their symptoms, 28 with suitable disease who refused to undergo exercise therapy, 24 who met exclusion criteria (5 unable to walk on treadmill, 4 with poor cardiorespiratory status, 4 critical limb ischemia, 9 aortoiliac disease,

**Table I.** Participant characteristics at baseline

| Variable | No. | Result |
|---|---|---|
| Age, mean (SD), years | 36 | 72 (10) |
| Sex | 36 | |
| Male, No. (%) | | 26 (72) |
| Female, No. (%) | | 10 (28) |
| BMI, mean (SD), kg/m$^2$ | 36 | 27.6 (4.1) |
| Current smokers, No. (%) | 25 | 10 (43) |
| Former smokers, No. (%) | 25 | 12 (52) |
| Pack-years, mean (SD) | 25 | 40.3 (24) |
| Diabetes, No. (%) | 27 | 7 (26) |
| Hypertension, No. (%) | 27 | 22 (81) |
| Dyslipidemia, No. (%) | 27 | 23 (85) |
| Ischemic heart disease, No. (%) | 27 | 8 (30) |
| AAA, No. (%) | 26 | 4 (15) |
| COPD, No. (%) | 25 | 2 (8) |
| ABI, mean (SD) | | |
| Lowest resting | 36 | 0.71 (0.21) |
| Lowest after exercise | 34 | 0.45 (0.33) |
| Asymptomatic leg | 17 | 0.98 (0.17) |
| Medications | | |
| ACE-I/ARA, No. (%) | 27 | 22 (81) |
| β-Blockers, No. (%) | 26 | 8 (30) |
| Aspirin, No. (%) | 26 | 21 (80) |
| Clopidogrel, No. (%) | 26 | 4 (15) |
| Statins, No. (%) | 27 | 21 (78) |

*AAA*, Abdominal aortic aneurysm; *ABI*, ankle-brachial index; *ACE-I*, angiotensin-converting enzyme inhibitor; *ARA*, angiotensin receptor antagonist; *BMI*, body mass index; *COPD*, chronic obstructive pulmonary disease; *SD*, standard deviation.

**Table II.** Laboratory values at baseline

| Variable | No. | Mean | SD |
|---|---|---|---|
| Hemoglobin, g/L | 34 | 143 | 16 |
| White cells, ×10$^9$/L | 34 | 7.3 | 2.3 |
| Neutrophils, ×10$^9$/L | 34 | 4.8 | 1.8 |
| C-reactive protein, mg/L | 33 | 6 | 24 |
| Creatinine, μmol/L | 33 | 79 | 19 |
| Albumin, g/L | 31 | 41 | 8 |
| Total cholesterol, mmol/L | 34 | 4.0 | 1.1 |
| HDL, mmol/L | 34 | 1.4 | 0.5 |
| LDL, mmol/L | 34 | 2.1 | 0.9 |
| Triglycerides, mmol/L | 34 | 1.4 | 0.7 |
| Homocysteine, μmol/L | 31 | 12.7 | 3.8 |
| $HbA_{1c}$, % | 26 | 6.1 | 0.9 |

*$HbA_{1c}$*, Glycosylated hemoglobin; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *SD*, standard deviation.

**Table III.** Reliability testing of custom region of interest (*ROI*) segmentation[a]

| ROI | *r* | Mean CoV (%) | ICC (95% CI) |
|---|---|---|---|
| Intraobserver | | | |
| Leg | 0.9993 | 0.3993 | 1.00 (0.999-1.000) |
| Thigh | 0.9970 | 0.8993 | 0.998 (0.996-0.999) |
| Interobserver | | | |
| Leg | 0.9978 | 0.544 | 0.999 (0.998-0.999) |
| Thigh | 0.9961 | 1.231 | 0.997 (0.989-0.999) |

*CI*, Confidence interval; *CoV*, coefficient of variation; *ICC*, intraclass correlation coefficient.
[a]$P < .0001$ for all tests.

1 warfarin therapy, and 1 large abdominal aortic aneurysm requiring surgery), 4 who participated in exercise therapy but did not consent to the study, and 1 patient who was later excluded after attending <80% of treadmill sessions. Data from the remaining 25 participants was combined with data from 11 participants from the treadmill-training arm who completed >80% of the training program and also underwent DEXA scans before and after training. Baseline demographic, laboratory data, and medications of the combined group are reported in Table I and Table II.

The modified ROI measurements described were highly reproducible within and between raters (Table III). All measurements demonstrated a Person correlation coefficient >0.99, a mean coefficient of variation <2%, and ICC ranging from 0.997 to 1.000 ($P < .0001$).

The main study outcomes are reported in Table IV. Walking outcomes—PFWD and 6MWD—significantly improved after the 12-week SEP (mean, 47 [SD, 76] m; $P = .001$) and (mean, 26 [SD, 50] m; $P = .004$). After the SEP, there was no significant change in whole-body SMM (mean change, −0.10 [SD, 0.72] kg; $P = .41$), in the LM of symptomatic calves (mean change, 9 [SD, 100] g; $P = .53$), or in the LM of asymptomatic calves (mean change, 35 [SD, 260] g; $P = .59$). In contrast, LM was significantly reduced in the thighs on both symptomatic (mean, −61 [SD, 220] g; $P = .043$) and asymptomatic sides (mean, −94 [SD, 120] g; $P = .005$).

No significant correlation was observed between changes in LM and changes in walking performance measures. Symptomatic-side thighs (ΔLM vs ΔPFWD: $r = -0.09$, $P = .62$; and ΔLM vs Δ6MWD: $r = -0.02$, $P = .91$), asymptomatic-side thighs (ΔLM vs ΔPFWD: $r = -0.25$, $P = .47$; and ΔLM vs Δ6MWD: $r = -0.5$, $P = .09$), symptomatic calves (ΔLM vs ΔPFWD: $r = -0.03$, $P = .87$; and ΔLM vs Δ6MWD: $r = -0.32$, $P = .058$), and asymptomatic calves (ΔLM vs ΔPFWD: $r = 0.52$, $P = .1$; and ΔLM vs Δ6MWD: $r = 0.31$, $P = .3$).

## DISCUSSION

The improvements in walking performance after supervised treadmill training are well documented[22] and were also observed in the present study of a typical claudicant population. In contrast, the effect of treadmill-based supervised exercise training on regional lower limb LM has not previously been investigated. Importantly, our

**Table IV.** Study outcomes[a]

| Variable | No. | Baseline, mean (SD) | 12-week, mean (SD) | Change, mean (SD) | *P* value |
|---|---|---|---|---|---|
| PFWD, m | 33 | 165 (78) | 213 (93) | 47 (76) | .001 |
| 6MWD, m | 36 | 395 (78) | 421 (68) | 26 (50) | .004 |
| Total body SMM, kg | 36 | 24.2 (6.2) | 24.1 (6.2) | −0.10 (0.72) | .41 |
| Calf LM | | | | | |
| Symptomatic, g | 55 | 2671 (610) | 2680 (590) | 9 (100) | .53 |
| Asymptomatic, g | 17 | 2701 (57) | 2736 (56) | 35 (260) | .59 |
| Thigh LM | | | | | |
| Symptomatic side, g | 55 | 5826 (1500) | 5764 (15) | −61 (220) | .043 |
| Asymptomatic side, g | 17 | 5684 (1400) | 5590 (1400) | −94 (120) | .005 |

*6MWD*, 6-minute walk distance; *LM*, lean mass; *PFWD*, pain-free walking distance; *SD*, standard deviation; *SMM*, skeletal muscle mass.
[a]Comparison between participants in the treadmill-only supervised exercise training.

data have shown that completion of such training is not accompanied by a loss of LM in the symptomatic calves of patients, generally considered the ischemic end organ in patients with infrainguinal arterial disease.[16,23]

In addition, no change in LM was observed in the asymptomatic calves of claudicant participants. This raises doubt on the hypothesis that the regional ischemia-reperfusion experienced by exercising claudicant individuals promotes skeletal muscle atrophy. Mass alone is probably not the only determinate of strength and function.[24] In support of this, changes in muscle fiber composition have been observed after supervised exercise. Beckitt et al[7] observed that 3 months of supervised treadmill training resulted in a proportional increase in myosin heavy chain type I expression in symptomatic calf muscles and that this correlated with improved walking performance.

Surprisingly, however, a small but statistically significant reduction in LM was observed in the thighs from both asymptomatic and symptomatic sides after the exercise program was completed. The cause and importance of this observation is currently unknown but is consistent with a previous randomized trial of exercise programs where patients lost strength in both lower limbs after crossing over to treadmill training from strength training.[13] Furthermore, it may explain why treadmill-only training programs fail to improve performance-based tests of function, such as chair stand, gait speed, and standing balance, which rely on proximal lower limb strength.[12]

In further support of a link between distal ischemia and proximal muscle groups is the observation that the strength of flexors and extensors, of hip and knee, are highly correlated with the severity of the peripheral ischemia, defined by ABI.[11] Moreover, biomechanical gait analysis of patients with intermittent claudication has demonstrated reduced hip, knee, and ankle power on commencement of walking, which worsens after the onset of pain but is also apparent on the unaffected side in unilateral disease.[25,26] Together, this suggests that despite symptoms predominantly localizing to calf muscles on the symptomatic side, major deficits are observed in more proximal muscles and contralateral limb muscles remote to areas of reduced arterial perfusion.

Possible mechanistic explanations for this effect may lie in systemic humoral pathways or neurogenic reflexes. In support of this, Koopman et al[27] performed electrophysiologic studies on patients with unilateral claudication and found evidence of neuropathy in both symptomatic and asymptomatic limbs. In addition, histologic and electrophysiologic examinations of muscle and nerves from ischemic and nonischemic limbs have revealed evidence of both sensory and motor nerve dysfunction.[28,29]

Whether exercise therapy can reverse these neuromyopathic gait and functional abnormalities, which have received little research attention to date, is currently unknown. England et Al[30] reported that mixed walking and strength training preserved calf muscle strength but did not worsen peripheral neuropathy. Conversely, Hiatt et al[31] demonstrated that although treadmill training increased peak exercise performance, it was associated with increased skeletal muscle denervation histologically. Whether dysfunction or denervation of nociceptor afferents also occurs is unknown but is an important consideration because the main justification of walking exercises is suppression of walking-induced pain. Nevertheless, it could be suggested that exercise therapy alone might not be sufficient to reverse the neuropathic changes observed in PAD and that treadmill training alone may be harmful despite improvements in walking tests. Combining these considerations, the findings of the present study challenge the notion that enough is known about the effects of supervised exercise training to settle the debate about the optimal treatment of claudication.

This study has some limitations. Firstly, this study focused on patients with claudication undergoing treadmill-only training because this is the most often recommended form of exercise. Without a control group, certainty that the changes observed would not also be

seen in healthy controls cannot be achieved. In addition, the observed results cannot be extrapolated to programs that have a strength-training element.

Secondly, daily physical activity of patients was not monitored outside of training; thus, we cannot be assured it did not decrease, despite all patients being instructed to maintain their normal activity levels. In addition, there was slight variation in the instructions given to patients regarding at what level of pain to cease exercise. This may have resulted in a more profound ischemia-reperfusion injury in some of the group and is indeed an area that requires more study.

Thirdly, the measurement of LM is an anatomic assessment that sheds little light on muscle performance and quality. It is possible that despite LM reductions, muscle performance improved through changes at the fiber level. In addition, the follow-up duration was short, and it is not possible to speculate whether the changes in muscle mass would eventually be observed in the calves or whether there were favorable or deleterious consequences.

Future studies should avoid focusing only on symptomatic muscles but should include assessment of the contralateral and proximal muscle groups, which are also affected by PAD. Also, a shift away from end points dependent on pain perception and broadening of outcomes to include strength and functional measures may help identify the optimal exercise prescription.[32] Finally, the paucity of data on long-term outcomes after exercise therapy needs to be addressed.

## CONCLUSIONS

In this study investigating regional lower limb LM in patients undergoing 12 weeks of standard treadmill training for intermittent claudication, no loss of calf lean muscle mass was observed, casting doubt on the hypothesis that repetitive ischemia-reperfusion prevents maintenance of calf SMM. In contrast, a significant decrease in bilateral thigh LM was observed even in patients with unilateral symptoms. Further work is required to determine whether these changes affect strength, functional performance, quality of life, and survival, taking into account the exercise modality and the limitations delineated above.

## AUTHOR CONTRIBUTIONS

Conception and design: SV, JS
Analysis and interpretation: SV, MM, JS
Data collection: SV, CD
Writing the article: SV
Critical revision of the article: SV, MM, CD, RA, JS
Final approval of the article: SV, MM, CD, RA, JS
Statistical analysis: SV
Obtained funding: SV, JS
Overall responsibility: JS

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*Research Article*

# A Comparison of Measures of Endothelial Function in Patients with Peripheral Arterial Disease and Age and Gender Matched Controls

**Richard B. Allan,[1,2] Simon V. Vun,[1,2] and J. Ian Spark[1,2]**

[1]*Department of Vascular and Endovascular Surgery, Flinders University, Bedford Park, SA 5042, Australia*
[2]*Department of Vascular and Endovascular Surgery, Flinders Medical Centre, Bedford Park, SA 5042, Australia*

Correspondence should be addressed to J. Ian Spark; ian.spark@sa.gov.au

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This study compared flow-mediated dilatation (FMD), peripheral artery tonometry (PAT), and serum nitric oxide (NO) measures of endothelial function in patients with peripheral artery disease (PAD) against age/gender matched controls. 25 patients (mean age: 72.4 years, M : F 18 : 7) with established PAD and an age/gender matched group of 25 healthy controls (mean age: 72.4 years, M : F 18 : 7) were studied. Endothelial function was measured using the % FMD, reactive hyperemia index (RHI) using PAT and serum NO ($\mu$mol). Difference for each method between PAD and control patients and correlation between the methods were investigated. FMD and RHI were lower in patients with PAD (median FMD for PAD = 2.16% versus control = 3.77%, $p = 0.034$ and median RHI in PAD = 1.64 versus control = 1.92, $p = 0.005$). NO levels were not significantly different between the groups (PAD median = 7.70 $\mu$mol, control median = 13.05 $\mu$mol, $p = 0.662$). These results were obtained in elderly patients and cannot be extrapolated to younger individuals. FMD and PAT both demonstrated a lower hyperaemic response in patients with PAD; however, FMD results in PAD patients were unequivocally reduced whereas half the PAD patients had RHI values above the established threshold for endothelial dysfunction. This suggests that FMD is a more appropriate method for the measurement of NO-mediated endothelial function.

## 1. Introduction

The vascular endothelium, the functional lining of blood vessels, plays a critical role in vascular homeostasis. Through the local production of nitric oxide (NO) the normal endothelium has the capacity to regulate vascular tone, coagulation, inflammatory cell adhesion, and vascular smooth muscle cell proliferation [1, 2]. Whereas normal endothelial function is thought to be atheroprotective, endothelial dysfunction occurs early in the development of atherosclerotic lesions and is characterised by a prothrombotic phenotype and reduced bioavailability of nitric oxide [1, 3]. Indeed endothelial dysfunction is increased in atherosclerotic conditions such as coronary heart and peripheral artery disease (PAD) and is an independent predictor of future cardiovascular events [4, 5].

Given its central role in the pathogenesis of atherosclerosis, endothelial dysfunction is of great interest to investigators studying interventions that may improve endothelial function in the hope that improvements may modify disease progression and future cardiovascular risk. There is some evidence that endothelial dysfunction in patients with PAD [4, 6] can be improved; however, there are a number of different methods available to measure endothelial function and it is not clear which is the most appropriate for patients with PAD.

In a majority of clinical studies endothelial function is typically quantified by measuring flow-mediated dilatation (FMD), which is defined as vasodilation of an artery in response to an increase in luminal blood flow and thus laminar shear-stress [7]. This is routinely achieved by inducing hyperaemia following a brief ischaemic stimulus and vasodilatation occurs primarily through the effects of NO [8]. Measurement of FMD, however, is operator dependent and requires considerable skill and experience. Peripheral artery

Table 1: Characteristics of the sample groups.

| | Controls ($n = 25$) | PAD ($n = 25$) | |
|---|---|---|---|
| Mean age (years) (SD), range | 72.40 (7.21), 59–85 | 72.36 (8.91), 58–90 | $p = 0.986$ |
| Gender: male : female | 7 : 18 | 7 : 18 | |
| Mean BMI (SD), range | 28.7 (5.19), 19.9–43.7 | 28.3 (5.18), 22.3–43.6 | $p = 0.803$ |

PAD: peripheral arterial disease, SD: standard deviation, and BMI: body mass index.

tonometry [9] (PAT) is a more recently developed alternative method of endothelial function measurement. PAT measures the ratio of baseline digital pulse wave amplitude at rest and after 5 minutes of brachial artery occlusion [10]. This method requires less training and experience and is largely automated making it an attractive alternative to FMD. While FMD and PAT both measure hyperaemic response, a surrogate for NO bioavailability, they are measured in different parts of the circulation (conduit and resistance vessels, resp.), with varying dependence on NO [11]. Furthermore, in our unit, we have observed a poor correlation between FMD and PAT [12].

Serum biomarkers have also been used to assess endothelial dysfunction; however, direct measurement of serum NO is problematic due to its highly reactive nature and short half-life. By using ELISA techniques to measure NO metabolites, nitrate, and nitrite, one can indirectly measure NO activity via serum or urine samples [13]; however, it remains unclear how this relates to other measures such as FMD and PAT.

While the above methods have previously been used to assess endothelial function in a number of different populations, a comparison of these three methods has not been performed in a group with PAD. The aim of this study was to investigate whether there was a difference between the PAD and control groups for each of the three measurement methods and whether there is a correlation between the methods.

## 2. Methods

*2.1. Participant Selection.* Data was collected from participants enrolled in two studies in which FMD, PAT, and venous blood collection were performed as part of the investigation of specific interventions.

The first study was investigating the effects on endothelial function of moderate dose fish oil on healthy participants with no evidence of PAD [14]. The second study was investigating the effects of two different exercise regimes on participants with intermittent claudication [15]. Baseline FMD and PAT tests and venous blood as part of these studies were used.

The sample populations consisted of 25 participants with symptomatic PAD (claudication, Rutherford classification 1–3) in the exercise intervention study and 25 controls, age and gender matched to the PAD group, from the fish oil intervention study. The age, gender, and BMI data for each group are presented in Table 1. The control group and PAD group were well matched with no significant difference for age, gender, or BMI between the groups. No participants were on nitrate-based medications. All patients with PAD were on statins (which were withheld for a 24-hour period prior to testing) while none were in the control group.

This analysis was limited to the baseline tests because of the nonindependent nature of follow-up measurements and the risk of confounding variables due to potential differing effects of the interventions on each test.

This study was approved by the Southern Adelaide Clinical Human Research Ethics Committee. All participants provided written informed consent for the measurement of FMD, PAT, and serum NO levels prior to commencement of data collection.

*2.2. Peripheral Arterial Tonometry.* Participants were instructed to fast for eight hours, refrain from caffeine, alcohol, and tobacco, and avoid exercise for eight hours prior to testing.

PAT tests were performed using an EndoPAT device (Itamar Medical Ltd., Caesarea, Israel), following the manufacturer's guidelines, in a quiet, dimmed, and temperature controlled room as previously described [12] with upper arm occlusion at 250 mmHg. The hyperaemic response was analysed using the device's proprietary software and the reactive hyperemia index (RHI) was calculated from pre- and post-occlusion measurements by the device software using the method previously described by McCrea et al. [10].

*2.3. Brachial Artery Ultrasound Flow-Mediated Dilatation.* Fifteen minutes after the completion of the PAT testing, FMD was performed by a single experienced operator with the participant supine and the right arm in a supportive cradle with the transducer held in a stereotactic stand, following accepted guidelines [16] using forearm occlusion as previously described [12]. Thirty seconds of baseline imaging and three minutes of post occlusion imaging were recorded to obtain baseline and the maximal hyperaemic response measurements. The brachial artery diameter was measured, during diastole, using automated edge detection software (Brachial Artery Analyser, MIA-LLC, Coralville, USA). The % FMD was calculated using the following equation:

$$\text{FMD (\%)} = \left[\frac{(\text{maximum diameter} - \text{baseline diameter})}{\text{baseline diameter}}\right] \times 100. \quad (1)$$

*2.4. Serum NO.* Peripheral venous blood was drawn into 4 mL serum separator evacuated tubes, allowed to clot for

Table 2: RHI, FMD, and NO results for age and gender matched controls and patients with PAD.

| | Controls ($n$ = 25) | PAD ($n$ = 25) | |
|---|---|---|---|
| RHI median, IQR | 1.92, 1.84–2.24 | 1.64, 1.34–2.01 | $p = 0.005$ |
| FMD% median, IQR | 3.77, 1.87–6.15 | 2.16, 0.59–4.17 | $p = 0.034$ |
| NO ($\mu$mol) median, IQR | 13.05, 5.58–19.06 | 7.70, 6.17–15.07 | $p = 0.662$ |

PAD: peripheral arterial disease, IQR: interquartile range, FMD: flow-mediated dilatation, RHI: reactive hyperaemia index, and NO: nitric oxide.

10 min, and then immediately centrifuged at 3,000 g for 7 minutes at 20°C. Serum was stored at −80 degrees Celsius and thawed for later total NO assay. Due to the instability and short half-life of NO, measurement was performed on the NO metabolites, nitrite ($NO_2^-$), and nitrate ($NO_3^-$). Nitrate was converted to nitrite using an enzyme nitrate reductase and the nitrite levels were then measured with a coloured azo dye product of the Griess reaction using visible light at 540 nm (Total Nitric Oxide Assay Kit; Pierce Biotechnology, Rockford, IL) [17]. The nitrite levels detected represented the total NO metabolites present in each sample and the kit is able to recover 98% of nitrate in serum. Results were expressed in $\mu$mol with a detection limit of 0.35 $\mu$mol and a CV of 5.3%.

*2.5. Testing of Reproducibility.* Test-retest reproducibility testing was performed for both FMD and PAT as described previously [12]. Brachial artery diameter intraclass coefficient (ICC) = 0.989 ($p < 0.001$) and coefficient of variation (CV) = 1.52%, FMD ICC = 0.884 ($p = 0.002$) and CV = 15.0%, and RHI ICC = 0.298 ($p = 0.304$) and CV = 19.3%. Intra-assay CV for serum total NO assays was 9.8%.

*2.6. Statistical Analyses.* Data were analysed using the SPSS for Windows statistical package version 20 (SPSS Inc., Chicago, IL, USA).

The mean, standard deviation (SD) and range were reported for age and body mass index (BMI) and Student's $t$-test was used to test for difference between controls and patients with PAD. Due to the nonparametric nature of the FMD, RHI, and serum NO results, data median and interquartile range (IQR) were reported and Mann-Whitney $U$ tests were performed to assess for differences in results between the controls and those with PAD.

Correlation between FMD, RHI from PAT, and NO was assessed using Spearman correlation coefficient.

Test-retest reproducibility was assessed using the intraclass correlation coefficient (ICC) and coefficient of variation (CV) for the first and second measurements in the group of healthy volunteers.

All tests were two-tailed and the level of statistical significance was set at $p < 0.05$.

## 3. Results and Discussion

*3.1. Results.* The results for each test of endothelial function are presented in Table 2. The distribution of results for each method in controls and patients with PAD are displayed in Figures 1, 2, and 3.

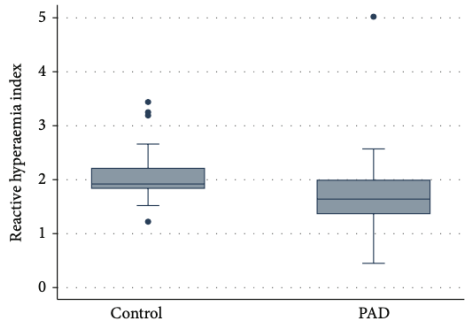


Figure 1: Plot of distribution of RHI values for age and gender matched controls and patients with PAD, $p = 0.005$.

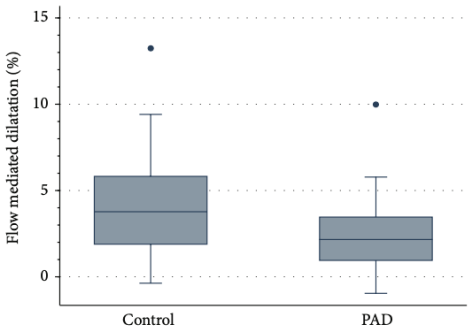


Figure 2: Plot of distribution of FMD % values for age and gender matched controls and patients with PAD, $p = 0.034$.

FMD and RHI were significantly lower in PAD patients compared to controls (FMD $p = 0.034$; PAT $p = 0.005$). There was no significant difference in NO levels between the two groups ($p = 0.662$).

No correlation was seen between the three tests of endothelial function in the participants with PAD (RHI versus FMD: $r = 0.182$, $p = 0.205$, RHI versus NO: $r = 0.034$, $p = 0.815$, FMD versus NO: $r = 0.07$, $p = 0.627$).

*3.2. Discussion.* To the best of our knowledge this is the first study to investigate the relationship of FMD, PAT, and serum NO metabolites in a sample of patients with PAD and to compare these three measures of endothelial function

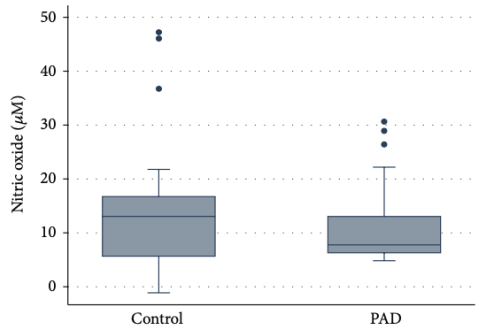


Figure 3: Plot of distribution of NO concentrations for age and gender matched controls and patients with PAD, $p = 0.662$.

between patients with PAD and a group controlled for age and gender. Both FMD and PAT measurements were significantly lower in this sample of PAD patients compared to age and gender matched controls; however, no significant difference was seen in results for serum NO metabolites.

The FMD results from the current study were markedly lower than that of the two previous studies of FMD in PAD patients and controls (5.40%–6.45% for PAD and 9.79%–12.80% for controls [3, 14] compared to 2.16% for PAD and 3.77% for controls in the current study). In part this may be due to differences in reporting of the data but it is also likely to be due to variations in technique as the two earlier studies used upper arm occlusion for FMD. This is known to be less dependent on NO-mediated dilatation and to produce a higher percentage dilatation [18] than the more widely accepted forearm method used in the current study. The current study is the first to demonstrate lower RHI in a sample of PAD patients compared to age and gender matched controls, indicating that the hyperaemic response is blunted in this patient group. However, the RHI measured in many of the PAD patients was high in relation to the previously reported threshold RHI value for endothelial dysfunction of 1.67 [9]. The median RHI of this group was 1.64 and based on the above threshold nearly half the PAD patients would be classified as having normal endothelial function. This is very surprising since this was a group with demonstrated symptomatic atherosclerotic disease and markedly lowered FMD values. It is known that NO-mediated vasodilatation is responsible for less than half the hyperemic response measured by PAT [19]. The relatively high RHIs measured by PAT in the PAD patients may relate to retention of the non-NO-mediated mechanisms that contribute to the hyperemic response as measured by PAT. This study highlights the need for studies to establish the underlying mechanisms of the hyperemic response in patients with PAD. These findings raise concerns that PAT may not be the most appropriate method for assessing NO-mediated endothelial function.

The lack of correlation between FMD and PAT in the current study is consistent with previous studies that use forearm occlusion for the FMD testing [12]. This is consistent with the evidence that these methods measure different aspects of the hyperemic response [18, 19].

The lower endothelial function found in PAD patients implies that there is the potential for improvement in endothelial function and this has been demonstrated in studies using FMD before and after surgical and endovascular interventions [20, 21]. The low FMD levels in the healthy controls (consistent with the known reduction in endothelial function with age [22]) suggest that the capacity for improvement in PAD patients may be limited and it is unclear if such mild improvements confer any long term health benefits related to reduced cardiovascular risk or delayed disease progression.

The lack of difference in serum measurement of NO metabolites may be due to the wide distribution in the NO results. This test is known to be highly sensitive to environmental factors [13]. Handling and analysis of samples were standardized in an effort to minimize these effects. In addition serum NO levels are affected by degradation associated with oxidative stress, which is expected to be higher in elderly patient with PAD. The high variability in results suggests that this may not be a robust test and may be of limited use in clinical studies.

A further limitation of this study is the variability of test results with all three methods. High variability of the NO results was found even with strict standardization of handling and analysis. FMD was found to have a better ICC and a CV than PAT. This is consistent with the only other report comparing reproducibility of the two methods [23]. It is well recognized that both of these methods have considerable test-retest variability. This appears to be intrinsic to the methods and relates to physiological variations rather than technical factors [24].

The use of vasoactive drugs is a potential source of variability; however, none of the participants were taking nitrate-based pharmacotherapy. All the PAD patients were on statins which are known to improve endothelial function [25]. As this study has demonstrated a reduction in endothelial function in these patients, the use of statins may, at worst, have decreased the observed reduction without affecting the overall conclusions.

A limitation of this study is that the endothelium-independent response of subjects was not assessed. This is feasible with FMD and allows assessment of both endothelial and nonendothelial mechanisms that may affect the hyperemic response. It is not feasible with PAT [26] and so comparison between these techniques could only be undertaken for endothelial-dependent vasodilatation.

Another potential limitation is that the participants were not recruited specifically for this study but were already enrolled in existing intervention studies. As data were restricted to preintervention baseline testing this should not have an influence on the reported results.

The small sample size of this study is also a limitation. However, even with this limitation there was a significant difference in FMD and RHI between PAD patients and controls.

## 4. Conclusion

This study has found that assessment of the hyperemic response by both FMD and PAT testing shows lower endothelial function in PAD patients when compared to age and gender matched controls. Serum NO results show a high degree of variation and did not demonstrate a significant difference between the groups. The difference in endothelial function between PAD patients and age-gender matched controls suggest an opportunity to improve endothelial function in PAD patients, though this may be limited by age dependent deterioration. Investigation is required into whether an improvement in endothelial function results in longer term health benefits in these patients.

An important limitation of these results that needs to be considered with this study is that the results were obtained in elderly patients with PAD and cannot be extrapolated to younger or less diseased individuals.

Both FMD and PAT are valid methods for assessing endothelial function in a research setting but the documented intertest variation makes them unsuitable for use in assessing individual patients in a clinical setting. If NO-mediated endothelial function is the phenomena of interest under investigation FMD would appear to be the more appropriate test as it is more purely NO-mediated in nature, demonstrates a more clear-cut reduction in patients with PAD, and has better reproducibility.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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