The Impact of Exercise on Immune Function, Inflammation and Microparticles in End Stage Renal Disease

By

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A Doctoral Thesis
Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

January 2019

Abstract

End-stage renal disease patients exhibit markedly elevated cardiovascular risk when compared to the general population, as well as chronic systemic inflammation as a result of aberrant immune system activation. Circulating microparticles are also elevated, driving both inflammation and thrombosis and further increasing cardiovascular risk. Regular, moderate intensity aerobic exercise may be a therapeutic strategy for the impaired immune function and pro-inflammatory environment seen in these patients, however previous studies investigating this effect have been methodologically limited. The studies included in this thesis aimed to characterise the impact of acute and regular aerobic exercise on immune function, markers of inflammation and microparticles in end-stage renal disease patients. It is hypothesised that aerobic exercise will have an anti-inflammatory impact on the immune system and cytokine environment and reduce circulating microparticle thrombotic potential.

To first characterise the microparticle response to acute aerobic exercise in healthy individuals for future comparison with patient populations, Study 1 (chapter 4) employed a randomised crossover design using 1-hour of moderate intensity (70% VO_2max) running exercise versus a no-exercise control trial in 15 healthy young males. Microparticles were characterised using flow cytometry from venous blood samples taken at baseline, immediately after exercise and 1.5-hours after exercise or rest. Data obtained by flow cytometry suggested that, whilst no changes were observed in the absolute number of circulating microparticle phenotypes, the proportion of platelet and neutrophil-derived microparticles that showed pro-thrombotic potential (by positively expressing tissue factor) was reduced in response to the exercise bout only and remained unchanged following rest.

Intradialytic cycling may be a more acceptable form of exercise for haemodialysis patients than more traditional independent exercise as it is completed during treatment time in the presence of healthcare professionals. Study 2 (chapter 5) employed a randomised controlled trial design to investigate the impact of a 6-month intradialytic cycling intervention on circulating inflammatory markers and microparticles, in comparison with a usual-care control condition in 40 haemodialysis patients (20 exercisers, 20 controls). Measures were completed at baseline and after 3 and 6 months of structured

exercise (or usual care control). Whilst the intervention had no impact on clinical results, physical performance or circulating chemokines, a reduction in the pro-inflammatory cytokine TNF-α was observed in the exercise group only. All other cytokines were either remained unchanged or were not detectable. No effects on MP characteristics were observed.

Low-frequency electrical muscle stimulation may be an alternative to dynamic exercise in HD patients that are too functionally limited to complete volitional exercise, as it mimics the local skeletal muscle contraction seen during dynamic exercise. Study 3 (chapter 6) also employed a randomised controlled trial design to investigate the impact of regular intradialytic low-frequency neuromuscular stimulation (n = 17) in comparison with regular intradialytic cycling (n = 15) and usual-care control (n = 16) on markers of systemic inflammation and measures of physical performance, over the course of 12 weeks. Outcome measures were completed at baseline and after the 12 week intervention. Whilst both the cycling and neuromuscular stimulation groups displayed improved scores in physical performance testing, there were no significant changes in any of the measured pro- or anti-inflammatory cytokines.

To investigate the impact of acute aerobic exercise in the immunosuppressed renal transplant population, Study 4 (chapter 7) investigated the effect of a single bout of moderate intensity walking exercise on immune cell phenotypes, markers of systemic inflammation and microparticles was investigated in renal transplant recipients (n = 15), in comparison with a non-dialysis dependent chronic kidney disease group (i.e. a uraemic control group – n = 16) and a non-CKD healthy control group (n = 16). In all three groups, the exercise elicited a reduction in the proportion of the monocyte pool that were deemed ‘intermediate’ and thus pro-inflammatory. The exercise bout also induced a reduction in the percentage of platelet-derived microparticles that positively expressed tissue factor in all three groups, however no other exercise-dependent effects were observed in immune cell subsets, markers of inflammation or microparticles. This form of exercise seems to be immunologically safe for renal transplant recipients.

Though some potentially anti-inflammatory effects of exercise were observed (TNF-α reduction in Study 2, intermediate monocyte reduction in Study 4) the aerobic exercise interventions measured here

largely had no impact on the inflammatory environment. This is encouraging as it suggests there was no aberrant immune system activation in response to exercise, and as such moderate intensity aerobic exercise participation should be considered immunologically safe in these patient populations and regular participation should be encouraged.

Key Words: End-stage renal disease, chronic kidney disease, aerobic exercise, systemic inflammation, immune function, cardiovascular disease, microparticles.

Acknowledgements

Throughout my PhD I have received help and support from so many people, all of whom deserve a great deal of thanks and gratitude.

Firstly, I am extremely grateful to have had such a supportive and motivational supervisor in Dr Lettie Bishop. She has supported me every step of the way throughout my PhD and I am enormously thankful for her contributions. Similarly, Dr David Stensel has provided fresh advice and support when needed and is always willing to help. At Leicester, Dr James Burton and Professor Alice Smith have given me excellent opportunities to work on fascinating and varied research studies, without which this thesis would not have been possible. Likewise, all of the members of the CYCLE-HD and Leicester Kidney Lifestyle Research Teams have helped me in many different ways. Additionally, Dr Naomi Martin has provided invaluable support and education in the lab, and without Sheila Estruel to help me analyse samples I would still be in that lab now so a huge thanks to Sheila.

My thanks also go to the clinical staff I have had the pleasure of working with. The nurses, doctors, healthcare assistants and admin staff have always been welcoming and supportive. Equally, I have had the privilege of working with a great number of patients during my PhD who have selflessly given up their time and energy. They have been source of inspiration and perspective and have made every long day worthwhile.

I must also acknowledge and thank the researchers who have contributed samples and data from their fascinating studies for inclusion in this thesis, primarily Dr Gordon McGregor and his research team at Coventry University and Fernanda Reistenbach-Goltz at Loughborough.

My friends and fellow researchers at Loughborough have always provided encouragement, and just the right amount of welcome distractions. My family's support during the highs and lows has been vital and is the reason I have made it this far. Lastly to Gaia, who has and continues to support me in every way possible – I cannot thank you enough.

Publications and presentations

The following publications and presentations have been derived from the work completed in this thesis:

Peer-reviewed publications

Patrick J. Highton, Naomi Martin, Alice C. Smith, James O. Burton, Nicolette C. Bishop. Microparticles and Exercise in Clinical Populations. *Exercise Immunology Review* 2018; 24: 46-58. (Narrative literature review).

Highton PJ, Neale J, Wilkinson TJ, Bishop NC, Smith AC. Physical Activity, Immune Function and Inflammation in Kidney Patients (the PINK Study): A Feasibility Study Protocol. *BMJ Open* 2017;7: e014713. (Full protocol for Study 4).

Scientific meeting presentations

Highton PJ, Stensel DJ, Goltz FR, Martin N, Bishop NC. Aerobic Exercise Reduces the Pro-thrombotic Potential of Circulating Microparticles in Healthy Individuals. Poster presentation, American College of Sports Medicine 'Exercise is Medicine' Conference, Minneapolis, USA, June 2018. (Derived from Study 1).

Highton PJ, Stensel DJ, Goltz FR, Martin N, Bishop NC. Aerobic Exercise Reduces the Pro-thrombotic Potential of Circulating Microparticles in Healthy Individuals. Poster presentation, United Kingdom Sports and Exercise Immunology Inaugural Conference, Loughborough, UK, April 2018. (Derived from Study 1).

Highton PJ, Martin N, Bishop NC, Smith AC. The Influence of Acute Aerobic Exercise on Microparticle Phenotype Counts in Renal Transplant Recipients. Poster Presentation, Renal Association UK Kidney Week Conference, Liverpool June 2017. (Derived from Study 4).

Highton PJ, Neale J, Churchward DR, Grantham CE, Bishop NC, Smith AC. The Influence of Acute Aerobic Exercise on Immune Cell Subsets in Renal Transplant Recipients. *Abstracts from the American Society of Nephrology Annual Conference 2016, Chicago, USA (November 2016).* (Derived from Study 4).

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Abbreviations

All abbreviations are defined within the main body of text in the first instance.

ACE – Angiotensin-Converting Enzyme

ANOVA – Analysis of Variance

APC – Antigen Presenting Cell

AVF – Arteriovenous Fistula

BIA – Bioelectrical Impedance Analysis

BIS – Bioelectrical Impedance Spectroscopy

BMI – Body Mass Index

CBA – Cytometric Bead Array

CCL – C-C Chemokine Ligand

CCR – C-C Chemokine Receptor

CD – Cluster of Differentiation

CHO – Carbohydrate

CKD – Chronic Kidney Disease

COPD – Chronic Obstructive Pulmonary Disease

CRP – C-Reactive Protein

CV – Coefficient of Variation

CVD – Cardiovascular Disease

CX_3CR – CX_3C Chemokine Receptor

DBP – Diastolic Blood Pressure

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

EMP – Endothelial Cell-Derived Microparticle

EPO – Erythropoietin

ES – Effect Size

ESRD – End Stage Renal Disease

ESWT – Endurance Shuttle Walk Test

FMD – Flow Mediated Dilation

GFR – Glomerular Filtration Rate

GMFI – Geometric Mean Fluorescence Intensity

HD – Haemodialysis

HDL – High-Density Lipoprotein

ICAM-1 – Intercellular Adhesion Molecule-1

IDC – Intradialytic Cycling

Ig - Immunoglobulin

IL – Interleukin

IP-10 – Interferon Gamma-Induced Protein-10

ISWT – Incremental Shuttle Walk Test

LDL – Low-Density Lipoprotein

LMP – Lymphocyte-Derived Microparticle

LPS – Lipopolysaccharide

MAP – Mean Arterial Pressure

MCP-1 – Monocyte Chemotactic Protein-1

MIG – Monokine Induced by Gamma Interferon

MMP – Monocyte-Derived Microparticle

MP – Microparticle

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NHS – National Health Service

NIHR – National Institute of Health Research

NMP – Neutrophil-Derived Microparticle

NO – Nitric Oxide

PD – Peritoneal Dialysis (CAPD – Continuous Ambulatory Peritoneal Dialysis)

PHA - Phytohaemagglutinin

PMP – Platelet-Derived Microparticle

PRO – Protein

PWV – Pulse Wave Velocity

RANTES – Regulated Upon Activation, Normal T Cell Expressed and Secreted

RCT – Randomised Controlled Trial

ROS – Reactive Oxygen Species

RPE – Rating of Perceived Exertion

RRT – Renal Replacement Therapy

RTR – Renal Transplant Recipient

SA – Staphylococcus Aureus

SBP – Systolic Blood Pressure

SD – Standard Deviation

SS – Shear Stress

STS – Sit-To-Stand

TGF – Transforming Growth Factor

TLR – Toll-Like Receptor

TNF – Tumour Necrosis Factor

T-reg – Regulatory T Cell

UHCW – University Hospitals of Coventry and Warwickshire

UHL – University Hospitals of Leicester

URTI – Upper Respiratory Tract Infection

VCAM-1 – Vascular Cell Adhesion Molecule-1

VO_2 – Oxygen Consumption

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Chapter 1

General Introduction

1.1 Thesis rationale and study structure

End-stage renal disease patients display chronically activated immune systems, both as a result of uraemic toxin build-up due to reduced renal clearance, and the pro-inflammatory impact of the treatments necessary to replace the function of the failed kidney (i.e. haemodialysis or renal transplantation). This aberrant immune system activation results in prolonged and unnecessary pro-inflammatory cytokine secretion, leading to a state of chronic systemic inflammation. Additionally, circulating microparticles are also elevated, driving both inflammation and thrombosis and increasing cardiovascular disease risk. Regular, moderate intensity aerobic exercise may be a therapeutic strategy for the impaired immune function and pro-inflammatory environment seen in these patients, however previous studies investigating this effect have been methodologically limited. Therefore, the studies included in this thesis have been designed to investigate the impact of either acute or regular aerobic exercise on markers of inflammation, microparticles and immune cell subsets in end stage renal disease patients.

This thesis includes four experimental studies. Firstly, in Study 1, the impact of an acute bout of moderate intensity running exercise on microparticle numbers and characteristics was investigated in young healthy males, in comparison with a no-exercise control trial completed in a randomised crossover fashion. This was in order to characterise the microparticle response to exercise in the general population, to allow subsequent comparison with patient groups in the subsequent studies.

Study 2 then investigated the impact of 6 months of thrice-weekly intradialytic cycling on microparticles and markers of systemic inflammation in haemodialysis patients, in comparison with a no-exercise usual care control condition. This exercise modality circumnavigates many perceived barriers to exercise participation in haemodialysis patients (e.g. lack of time to exercise, safety concerns) as it is completed during routine treatment hours in the presence of healthcare staff. This study 2 was completed on a cohort derived from a large, multi-centre randomised controlled trial.

Study 3 investigated the impact of 12 weeks of thrice-weekly low-frequency electrical muscle stimulation on markers of systemic inflammation in haemodialysis patients, in comparison with an

intradialytic cycling condition and a usual care control group. This exercise modality, whilst not being a true exercise mimetic, can mimic the local skeletal muscle contraction seen in aerobic exercise by using frequent electrical impulses targeted to a specific muscle group, and therefore can provide an alternative for patients who are too functionally impaired (e.g. due to orthopaedic impairments or extreme frailty) to complete dynamic exercise. This study employed a randomised controlled trial design.

Finally, renal transplant recipients do not require dialysis treatment, however they must take immunosuppressive medication in order to prevent rejection of their new kidney. There is no research to date that has investigated how aerobic exercise impacts the immune system and inflammatory environment in this population. This is highly pertinent as any change in immune function could increase the risk of infection (if immune function is further suppressed) or allograft rejection (if immune function is aberrantly activated). Therefore, Study 4 investigated the effect of an acute bout of moderate intensity walking exercise on immune cell subsets, markers of systemic inflammation and microparticles in renal transplant recipients, in comparison with a pre-dialysis chronic kidney disease group (to act as a non-immunosuppressed uraemic control group) and a healthy control group.

Taken together, these studies will provide evidence for the impact of both acute aerobic exercise and regular aerobic exercise training on the inflammatory environment in end-stage renal disease patients. Additionally, the results will provide information concerning the immunological safety of aerobic exercise in these patients, which is of particular importance in the renal transplant population. These findings will add to the current body of evidence investigating exercise and inflammation in end-stage renal disease, with a hope to informing future exercise guidelines in this population.

Chapter 2

Literature Review

2.1 Chronic Kidney Disease

Chronic kidney disease (CKD) is defined as abnormalities in kidney structure or function present for at least 3 months with implications for health. CKD is classified based on cause, glomerular filtration rate (GFR – the filtration capacity of the renal system and categorised as G1 - G5) and albuminuria category (CGA – categorised as A1 – A3).

The stages of CKD as assessed by GFR decline and albuminuria presence (albumin build-up begins as renal function declines) are presented in Figure 2.1. As renal function worsens, GRF reduces and albuminuria levels increase – this occurs in line with worsening CKD prognosis.

				Albuminuria		
				A1	**A2**	**A3**
				Normal/mildly increased	Moderately increased	Severely increased
				<30 mg/g	30-300 mg/g	>300 mg/g
GFR (mil/min/1.73m^2)	**G1**	Normal	≥ 90			
	G2	Mildly decreased	60 - 89			
	G3a	Mildly/moderately decreased	45 - 59			
	G3b	Moderately/severely decreased	30 - 44			
	G4	Severely decreased	15 - 29			
	G5	Kidney failure	< 15			

Figure 2.1. The Kidney Disease Improving Global Outcomes (KDIGO) Work Group classification criteria for CKD severity. (KDIGO Working Group, 2013). Darker colour = worse prognosis.

GFR decline is categorised in increasing severity from G1 to G5, and albuminuria is classified in increasing severity from A1 to A3. Once renal function has declined to the point that it can no longer sustain life (i.e. stage G5), End-Stage Renal Disease (ESRD) has been reached and a form of Renal Replacement Therapy (RRT) must be implemented. The speed of progression through the CKD stages is determined by a number of factors, including original diagnosis and prognosis, disease management protocol (i.e. medication and treatment compliance) and lifestyle factors such as physical activity and diet. Generally, however, progression is slow, and most patients never reach the point of requiring RRT to survive (Din, Salem, and Abdulazim 2016). As the renal system serves a variety of functions (water and electrolyte balance regulation, waste product filtration, hormone secretion and acid-base balance

maintenance), renal failure in ESRD is characterised by many profound complications including uraemia, hyperkalaemia, severe acidosis and oedema.

Renal Replacement Therapy

The goal of RRT is to artificially replace the function of the renal system to maintain and support life. This can be achieved through either implementing one of several forms of dialysis, or through renal transplantation.

Dialysis is a procedure which artificially carries out the primary functions of the kidneys, i.e. removal of uraemic toxins and excess water (ultrafiltration). In haemodialysis (HD), this is accomplished by removing venous blood through a surgically created arteriovenous fistula (AVF) or permanent chest or femoral catheter ('perma-cath') and passing it through an extracorporeal circuit to a dialyser, where the unwanted toxins and water are removed across a semi-permeable membrane and discarded (blood cells and proteins cannot cross this membrane). Dialysate (dialysis fluid designed to aid in the removal of uraemic toxins) flows in the opposite direction to the blood within the dialyser, thus creating a gradient to elicit maximum dialysis efficacy. Erythropoietin and bicarbonate are also administered during this process, to correct anaemia and acidosis, respectively, both of which are caused by diminished renal function. Current guidelines dictate that in 'conventional' HD, this process is completed gradually over a 4 hour treatment, three times a week (either Monday, Wednesday and Friday or Tuesday, Thursday and Saturday – the extra day off at the end of each week is referred to as the 'long break') at a dialysis unit (Rocco et al. 2015). HD can also be completed overnight (usually for 6-7 hours) at some HD units or at home at the convenience and competence of the patient.

Another option for RRT is peritoneal dialysis (PD). This process involves the administration of dialysate into the abdomen, whereby the peritoneum acts as a semi-permeable membrane across which uraemic toxins and excess water can travel. The fluid is left in the abdomen for several hours and then emptied and discarded. This process is repeated manually a predetermined number of times a day at the patient's convenience or accomplished throughout the night via an automated machine.

Finally, the so called 'gold standard' form of RRT with regards to patient outcome and treatment burden is renal transplantation (Tonelli et al. 2011). A donor kidney (usually from a living relative or deceased donor) is transplanted into the renal transplant recipient (RTR), thus replacing the function of the failed kidneys - this then negates the need for regular dialysis treatment. However, regular immunosuppressive medication must then be administered to prevent allo-immunity, i.e. an immune response to non-self-antigens from the transplanted kidney by the acquired immune system. This immunosuppressive medication can be administered in a variety of formats, the functions and associated side-effects of which are summarised in Table 2.1. The specific effects of renal transplantation and the associated medication on immune function will be discussed later in this chapter. However, demand of donor kidneys is far greater than supply, meaning the majority of ESRD patients undergo HD treatment whilst on a transplant waiting list.

Due to the barriers that hamper recruitment of PD patients in the University Hospitals of Leicester Trust, which include small patient numbers (4.9% of RRT patients in this region vs 41.9% for HD and 53.1% for transplant) and infrequent and irregular appointment times, no PD patients were recruited into the studies presented in this thesis. As such, PD and the associated literature will only be discussed where necessary and not described in detail.

Type of Drug	Examples	Function/s	Side Effects
Corticosteroids	Prednisone, Prednisolone, Methylprednisolone	Reduce antigen presenting capacity of APCs	Weight gain, hypertension, hypercholesterolaemia, accelerated atherosclerosis, diabetes mellitus,
Calcineurin Inhibitors	Tacrolimus, Cyclosporine	Prevent antigen presentation-induced activation and proliferation of T lymphocytes	Hypertension, hypercholesterolaemia, hyperglycaemia, hypomagnesia, hyperkalaemia, hyperuricaemia
Purine Antagonists	Mycophenolate mofetil, Azathioprine	Reduce DNA and RNA synthesis in the production of T lymphocytes	Weight gain, hypertension, hyperglycaemia
Mammalian Target of Rapamycin (mTOR) Inhibitors	Sirolimus, Everolimus	Prevent the activation of protein translation within lymphocytes	Proteinuria, hepatotoxicity, leukopenia, anaemia, dyslipidaemia

Table 2.1. Summarising the various forms of immunosuppressive medication regularly used by renal transplant recipients. Often a combination of more than one drug is used ('combination therapy') to elicit adequate immunosuppression.

Incidence and Prevalence of CKD

CKD and the subsequent ESRD is a growing health issue both in the UK and globally, characterised by progressively increasing incidence and prevalence rates and consequential increases in health care costs. In the UK, the incidence of patients receiving RRT decreased from 120 pmp (per million population) in 2015 to 118 pmp in 2016, though this actually resulted in an extra 7,759 patients receiving RRT (due to population increases). Of these incident patients, 60.66% were receiving HD, 19.6% were receiving PD and 9.3% received a functioning renal transplant whilst 4.6% died or stopped treatment between 2015 and 2016 (Hole et al. 2018).

Similarly, the number of prevalent RRT patients in the UK in 2016 was 962 pmp, an increase of 3.9% from 2015 (and a 44.4% increase from 2000). From 2015, this represented a 0.9% increase in HD patients, a 5.1% increase in graft-functioning transplant patients, and a 0.1% decrease in PD patients (MacNeill et al. 2018).

Causes of and Risk Factors for CKD

CKD can be initiated by a variety of causes, both lifestyle-mediated and genetic. Table 2.2 summarises the frequency of the main primary diagnoses of prevalent RRT patients in the UK in 2016 that initiated their renal insufficiency and resulted in ESRD.

Primary Diagnosis	Number	% of all Patients
Glomerulonephritis	11,716	19.1
Diabetes	10,375	16.9
Pyelonephritis	6,344	10.3
Polycystic Kidney	6,146	10.0
Hypertension	3,774	6.1
Renal Vascular Disease	1,809	2.9
Other	10,114	16.5
Aetiology Uncertain	9,274	15.1

Table 2.2. Summarising the primary diagnoses of prevalent RRT patients in the UK in 2016. Often, as seen from the table, the aetiology is uncertain. A renal biopsy is sometimes necessary to diagnose these patients, and as knowing the cause is sometimes unnecessary as the treatment is the same regardless (barring any other significant complications), the risk of a biopsy is not thought to be worth obtaining a diagnosis. Adapted from MacNeill et al. (2018).

Regardless of cause, the consequence of all of the above conditions is the same – progressive and irreversible destruction or obstruction of the nephrons (the 'functional units' within the kidney – there are roughly 1 million nephrons in a single adult kidney) resulting in impaired renal function.

Besides the causes mentioned above, there are also a large variety of risk factors that may not cause CKD directly, but can increase the risk of CKD initiation and stimulate progression. These risk factors can be classified as either modifiable or non-modifiable and are summarised in Table 2.3.

Non-Modifiable Risk Factor	Modifiable Risk Factor
Age	Obesity
Male sex	Hypertension
Family history of CKD	Dyslipidaemia
Genetic predisposition	Diabetes mellitus
Ethnicity	Smoking
Low birth weight	Alcohol consumption
	Nephrotoxic drug consumption
	Infection
	Autoimmune diseases
	Proteinuria
	High uric acid levels

Table 2.3. The major modifiable and non-modifiable risk factors for CKD incidence (Kazancioğlu 2013; Yamagata et al. 2007).

2.2 Cardiovascular Disease in CKD and ESRD

Cardiovascular Disease (CVD) is highly prevalent in the CKD population. Within the spectrum of CKD, CVD includes ischaemic heart disease, congestive heart failure, arrhythmias and peripheral vascular disease (Subbiah, Chhabra, and Mahajan 2016). In CKD stages G4 and G5, it has been reported that over 60% of patients have some form of CVD (Foley 2010). It has been suggested that any incident (i.e. newly diagnosed) CKD patient is 20 times more likely to die a CVD-related death than progress to ESRD and require RRT (Kundhal and Lok 2005), and CVD death rates in ESRD patients are 20-40 times higher than in the general population (Collins, Li, and Herzog 2001). CVD is the leading cause of death in RRT patients in the UK, representing 19-26% of all deaths dependent on RRT modality and vintage (Steenkamp, Pyart, and Fraser 2018).

Whilst the high prevalence of CVD in ESRD can be partly attributed to increased prevalence of traditional cardiovascular risk factors (dyslipidaemia, obesity, hypertension and diabetes) (Chen et al. 2016), the nature of these relationships may differ to those seen in the general population. A reverse epidemiology has been observed in ESRD patients, suggesting that some CVD risk factors that would signal increased CV risk in the general population may actually offer a survival benefit in ESRD patients. For instance, having a higher BMI may actually be cardio-protective in ESRD, as increased BMI is associated with greater survival in this population (Park et al. 2014) – this has been termed the 'obesity

paradox'. This may be due to the interaction between protein-energy wasting (PEW) and malnutrition-inflammation-atherosclerosis (MIA) syndrome, both of which can increase mortality in ESRD (Kalantar-Zadeh et al. 2003; De Mutsert et al. 2008). PEW is characterised by reduced serum nutritional parameters (albumin, cholesterol), reduced body mass (reduced fat mass, reduced energy or protein intake), and reduced muscle mass (cachexia, sarcopenia) (Fouque et al. 2008). MIA is characterised by the co-existence and interaction between malnutrition, systemic inflammation and atherosclerosis (Zyga, Christopoulou, and Malliarou 2011). Inflammation links PEW and MIA, creating a vicious cycle that can drastically increase mortality risk. The increased survival seen in overweight/obese patients may therefore be due to a reduced likelihood of PEW/MIA development, as they are unlikely to be undernourished (Park et al. 2014). Furthermore, the 'traditional' long term effects of obesity (e.g. cardiovascular disease, diabetes) on mortality may be outweighed by the short term health risks of PEW/MIA, which can cause death much more rapidly (Park et al. 2014) and as such obesity elicits greater long-term survival. Regardless of the cause, the primary mechanism through which CVD influences mortality in CKD and ESRD is increased arterial stiffening resulting in increased intima-media thickness and calcification, reducing the ability of the vasculature to dampen elevated BP and thus increasing cardiac strain. The primary cause of this stiffening is chronic, low-grade systemic inflammation (London 2018), as explained below.

2.3 Inflammation in ESRD

A 'non-traditional' risk factor that may promote both cardiovascular and all-cause mortality in ESRD patients is systemic inflammation. Under normal conditions, inflammation is a protective response to infection or injury that serves to minimise damage and allow efficient healing. The purpose of inflammation is to isolate and remove the cause of the inflammation as well as deliver the necessary cells and soluble proteins necessary to initiate healing. Under normal circumstances, inflammation is a transient response that dissipates when no longer necessary. Leukocytes, cytokines, chemokines and the complement and coagulation systems co-ordinate to eliminate the source of inflammation, and then are removed accordingly.

However, in chronic inflammatory conditions, the inflammatory response becomes unregulated and may not diminish when appropriate, and as such becomes chronic low-grade systemic inflammation. This is either because of an inadequate anti-inflammatory response or aberrant immune activation resulting in continued production of inflammatory mediators. It is characterised by a persistent, systemic increase (at least 2-fold) in many pro-inflammatory mediators (e.g. interleukin (IL)-6, IL-17a, tumor necrosis factor-alpha (TNF-α)) and a subsequent increase in acute phase reactants (e.g. C-Reactive Protein – CRP, which is produced by hepatocytes in response to elevated circulating concentrations of pro-inflammatory cytokines such as IL-6 and IL-1β). This dysregulated inflammation is a pathophysiological phenomenon. Systemic inflammation is often quantified by measuring circulating CRP levels – in the healthy population CRP levels are usually between 1-3 mg/L, whereas the mean CRP level in the ESRD population is 7-8 mg/L (Carrero and Stenvinkel 2010).

Causes of Inflammation in ESRD

Systemic inflammation in ESRD patients can be a consequence of both ESRD *per se* and a side-effect of RRT. These causes and sources of inflammation are listed in Table 2.4.

Consequence of ESRD	**Consequence of RRT**
Uraemic toxin build-up	Bio-incompatibility of dialysis membrane and dialysate
Chronic infections	Endotoxin exposure from extracorporeal circuit or impure dialysate
Volume overload	Drug regime (e.g. EPO, Iron)
Reduced clearance or increased synthesis of pro-inflammatory mediators	Infection at vascular access site
Excessive ROS production	High glucose load of peritoneal dialysis fluid
Hyperlipidaemia	Back-filtration of impurities
Detrimental changes in body composition (increased white adipose mass, decreased skeletal muscle mass)	
Sympathetic over-activity	
Genetic heritability and epigenetic regulation	

Table 2.4. The major sources of inflammation in ESRD patients, as a consequence of either ESRD of RRT *per se*. (Amore and Coppo 2002; Carrero and Stenvinkel 2010).

The ESRD-specific causes of inflammation are more numerous and seemingly more potent than those specific to RRT – this is evidenced by the high incidence of elevated pro-inflammatory biomarkers observed in CKD stage G5 patients not yet receiving RRT (Barreto et al. 2010).

The overall consequence of the causes listed in Table 2.4 is aberrant immune activation resulting in both stimulated pro-inflammatory cytokine release (Grabulosa et al. 2018) and dampened or impaired anti-inflammatory cytokine release, creating chronic low-grade systemic inflammation. The effects of ESRD and RRT on the immune system and the subsequent effects on the cytokine environment are discussed later in this chapter.

Consequences of Inflammation in ESRD

Elevations in pro-inflammatory cytokines (e.g. IL-6, TNF-α) and acute phase reactants (e.g. CRP) in ESRD patients have been associated with increased risk of hospitalisation (Ikizler et al. 1999) and increased mortality (Barreto et al. 2010; Jovanovic et al. 2015). Due to the systemic nature of unregulated inflammation in this population, the mechanisms via which it can promote morbidity and mortality are numerous and are explained below.

Elevated pro-inflammatory mediators can initiate and stimulate muscle protein breakdown in ESRD patients. In the HD population, elevated CRP levels have been associated with reduced muscle protein synthesis and increased muscle protein breakdown, resulting in a strong inverse correlation between CRP concentration and net protein balance (Deger et al. 2017). In CKD and HD patients, systemically elevated TNF-α can both accelerate activation of the caspase-3 proteolytic pathway and suppress insulin receptor signalling within skeletal muscle, thus promoting net muscle protein breakdown (Chen et al. 2013). As mentioned above when discussing MIA syndrome, skeletal muscle wasting can greatly increase mortality in this population.

Pro-inflammatory cytokines can also have potent effects on the vasculature of ESRD patients. Elevated pro-inflammatory cytokine (IL-1, IL-6, TNF-α) levels have been associated with vascular stiffness (as measured by pulse wave velocity - PWV) (Desjardins et al. 2017; Nowak et al. 2016), endothelial

dysfunction (as measured by flow-mediated dilation) (Mitsides et al. 2017) and atherosclerosis (Hu et al. 2016). The mechanisms behind the influence of inflammation on the vasculature are complex and multifaceted. Briefly, elevated levels of pro-inflammatory mediators can increase endothelial cell adhesion molecule (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-Selectin etc.) expression and intima-media chemokine (monocyte chemotactic protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), IL-8) expression, which increases leukocyte tethering to activated endothelial cells and subsequent migration into the surrounding tissue. Supporting this, ESRD patients display elevated adhesion molecule and chemokine (ICAM-1, VCAM-1, MCP-1) levels (Papayianni et al. 2002) which have been positively associated with carotid atherosclerosis in this population (Papayianni et al. 2003). Once tethered, the leukocytes extravasate into the intima/media of the artery and thus promote atherosclerotic plaque formation (particularly in the case of macrophages which become lipid-laden foam cells through a net uptake of oxidised low-density lipoprotein (LDL) particles via increased scavenger cell receptor expression). The 'shoulder (i.e. the region of vasculature immediately surrounding the bulging lipid core)' of an atherosclerotic legion typically consists of activated macrophages, T-lymphocytes and mast cells. The cellular activation is caused by ligation of the antigen receptor by oxidised LDL particles. Once resident within atherosclerotic plaque, these activated leukocytes release inflammatory mediators (e.g. IL-6, TNF-α, interferon-gamma (IFN-γ)) and proteolytic enzymes, propagating a local inflammatory environment that reduces plaque stability (via increased matrix metalloproteinase expression which catabolizes the collagen in the plaque shoulder) and increases the chances of plaque rupture and subsequent thrombus formation, through which the majority of complications associated with atherosclerosis are initiated (e.g. stroke, myocardial infarction) (Hansson 2003; Libby, Ridker, and Maseri 2002). Additionally, the production of pro-inflammatory chemokines (e.g. MCP-1, RANTES) by tubular epithelial cells within the kidney has been associated with the initiation and progression of renal injury and hence declining renal function (Anders, Vielhauer, and Schlöndorff 2003).

Oxidative stress (OS) may also link inflammation and CVD. As renal function declines, markers of OS (e.g. malonyldialdehyde, plasma F_2-isoprostanes) increase in the circulation (Dounousi et al. 2006). This may

be the consequence of increased reactive oxygen species (ROS) production, as increased NAD(P)H oxidase activity has been reported in patients with renal insufficiency without a concomitant increase in antioxidant capacity (Karamouzis, Sarafidis, and Karamouzis 2008). Increasing OS promotes endothelial dysfunction by reducing nitric oxide (NO) availability – this alters vascular permeability and allows LDL cholesterol to migrate into the intima. This LDL cholesterol is then oxidised, upon which it activates leukocytes as explained above, promoting their secretion of pro-inflammatory mediators (Cachofeiro et al. 2008).

Additionally, systemic inflammation has been associated with depression in ESRD patients (Kalender, Ozdemir, and Koroglu 2006). The exact nature of this relationship is unclear; however, pro-inflammatory cytokines (e.g. IL-6, IFN-α) have demonstrated the ability to cross the blood brain barrier and interact with the central nervous system, therefore altering neurotransmitter systems (e.g. by reducing serotonin and/or dopamine secretion) and thus inducing depressive symptoms (e.g. fatigue, anxiety) (Felger and Lotrich 2013). Depression has been strongly associated with mortality in the ESRD population (Farrokhi et al. 2014).

Lastly, systemic inflammation can be both a cause and consequence of immune system dysfunction in ESRD, resulting in increased infection rates in ESRD patients as explained below. Due to the varied and numerous pathophysiological mechanisms promoted by elevated systemic inflammation as explained above, the assessment of multiple inflammatory markers in ESRD patients has proven highly predictive for all-cause mortality (Jung et al. 2018). This is likely due to the systemic nature of chronically elevated inflammation. As mentioned above, chronic systemic inflammation has shown the ability to drive a number of pathophysiological processes, including skeletal muscle wasting, cardiovascular disease and depression. As such, it is unsurprising that chronic systemic inflammation is predictive of all-cause mortality given the myriad of pathophysiological influences it can exert.

2.4 Immune Dysfunction in ESRD

The characteristics and functions of the immune system are widely and detrimentally impacted by ESRD. This is primarily due to both the uraemia caused by diminished or absent renal function and the RRT modality implemented to partially reverse uraemia.

Uraemia

The studies discussed in this section were either completed in pre-dialysis uraemic CKD patients or completed in vitro in a uraemic environment – this is noted as each study is discussed. Firstly, pathogen recognition is reduced in a uraemic environment; lipopolysaccharide-stimulated $CD14^+$ monocyte toll-like receptor 4 (TLR4) expression in uraemic CKD patients is significantly reduced when compared to healthy controls (Ando et al. 2006). This also leads to a reduction in stimulated monocyte TNF-α, IL-1β, IL-6 and IL-8 synthesis that has been associated with increased infection rates, highlighting the potential clinical importance of this detriment. However, conversely, recent work has shown that skeletal muscle tissue isolated from CKD patients displays increased TLR4 gene and protein expression, which is associated with increased TNF-α gene expression (Verzola et al. 2016). The cause for this disparity is unclear but may be due to the disparity of assessing serum vs muscle tissue and requires further investigation. It may be that these both represent a pathophysiological effect – reduced TLR4 expression by circulating monocytes may impair antigen recognition, whilst increased TLR4 expression within skeletal muscle may promote an inflammatory environment, which has been observed in insulin resistant individuals (Reyna et al. 2008), and thus promote muscle wasting. However, this theory has not, to the author's knowledge, been investigated in the ESRD population and thus remains speculative. Granulocyte function is also impaired. When *p*-cresol sulfate (a uraemic toxin) was incubated at levels 'typically seen in ESRD' (5-30 μg/mL, although 30 μg/mL may actually be considered higher than the usual range seen in more recent studies (Duranton et al. 2012; Guida et al. 2013, 2014; Nakabayashi et al. 2011)) with blood taken from individuals with normal renal function (estimated GFR of >80ml/min/1.73m^2), granulocyte glucose breakdown and respiratory burst activity were significantly reduced, an effect which was strengthened with increased toxin concentration and incubation time

(Vanholder et al. 1995). Likewise, granulocyte inhibitory protein-1 (GIP-1), which is present in uraemic blood, can decrease granulocyte deoxyglucose uptake, chemotaxis, oxidative metabolism and intracellular killing capacity (Haag-Weber and Hörl 1996). Other uraemic toxins, such as glycated serum proteins (Cohen, Rudnicki, and Horl 2001) and leptin (Ottonello 2004) can interfere with neutrophil chemotaxis and increase rates of apoptosis, and as such neutrophils harvested from uraemic patients show higher rates of apoptosis and reduced rates of superoxide production when compared to non-uraemic controls (Cendoroglo et al. 1999). Conversely, neutrophils extracted from uraemic patients display increased basal levels of extracellular trap formation, suggesting a 'pre-activation' of these cells (Kim et al. 2017). Lastly, uraemia may skew the apoptosis-necrosis balance in neutrophils towards necrosis, thus creating an inflammatory environment in the surrounding tissue due to the release of proteolytic enzymes originally contained in the cytoplasm (Glorieux, Vanholder, and Lameire 2003).

Uraemia can also influence other monocyte functions. Monocytes cultured with uraemic serum from HD patients displayed a reduction in both endocytosis and CD80/86 expression when compared to those cultured in healthy serum, suggesting that monocyte phagocytosis and antigen presentation capabilities were compromised (Lim et al. 2007). Monocyte differentiation into dendritic cells was also impaired in uraemic serum (Lim et al. 2007). As such, ESRD patients display reduced circulating dendritic cell numbers, as well as reduced CD80/86 and HLA-DR expression (Agrawal et al. 2009), suggesting an impaired ability to both present antigen to and activate T lymphocytes. Uraemia also alters monocyte subset distribution, increasing the proportion of the non-classical, pro-inflammatory $CD16^{+}$ subset (Brauner et al. 1998). Monocyte ICAM-1 expression is also increased, promoting endothelial adhesion and ultimately macrophage infiltration, foam cell formation, and acceleration and destabilisation of atherosclerotic plaque. Conversely, the uraemic toxin indoxyl sulphate can increase LPS-stimulated macrophage NO release when incubated at levels typically seen in ESRD (62.5-1000 µM) (Adesso et al. 2013), which would be expected to promote endothelial health. However, this was mirrored by increased macrophage superoxide release, and therefore may be a response to immune over-activation. Accordingly, indoxyl sulphate can promote monocyte differentiation into pro-inflammatory M1 macrophages with greater IL-6 and MCP-1 expression (Barisione et al. 2016), as well as increase the rate

of apoptosis of endothelial cells and ultimately damage the endothelium (Kim et al. 2017). Finally, when monocyte-derived macrophages were incubated with *p*-cresol sulfate at 'low' (10 μg/mL) or 'high' (50 μg/mL) concentrations, NO production and phagocytic capacity (measured by LPS-stimulated bead engulfment) was increased in the low concentration condition, whilst the high concentration condition promoted increased expression of antigen presenting cells (CD80, CD86, HLA-DR) on the macrophages (Azevedo et al. 2016).

Uraemia can both directly and indirectly affect the adaptive immune system. Indirectly, cytokine-mediated stimulation of the adaptive immune system is altered, which subsequently alters the adaptive response. Depending on the study in question, uraemia has been shown to cause either an increase in monocyte IL-12 secretion and hence predomination of Th1-activated T-lymphocyte cell-mediated immunity (Girndt et al. 2001), or an increase in Th2 IL-4 secretion, which can inhibit Th1-mediated immunity via IFN-γ suppression and thus create predomination of Th2-activated B-lymphocyte humoral immunity (Libetta, Rampino, and Dal Canton 2001). The cause for this disparity is unknown. Uraemia can also more directly affect cells of the adaptive immune system. $CD3^+$ T cells cultured in uraemic serum displayed reduced phytohaemagglutinin (PHA)-stimulated proliferation, increased CD95 expression (a marker of cellular apoptosis), and increased CD45RO expression (a memory T cell marker); this suggests a more terminally differentiated 'aged' immune system (Moser et al. 2003). The magnitude of the depletion of naïve and central memory $CD4^+$ and $CD8^+$ T cells is directly related to the degree of uraemia, as well the severity of oxidative stress, secondary hyperparathyroidism, iron overload and inflammation and is also strongly related to increased infection risk (Vaziri et al. 2012; Yoon et al. 2006). Uraemia also detrimentally affects regulatory T cells (T-regs). Uraemic patients display a reduced circulating T-reg number and cytokine secretion (IL-10 and TGF-β1) (Zhang et al. 2010), limiting their suppressive, anti-inflammatory and self-regulatory functions and promoting immune over-activation. This reduction also inversely correlates with circulating interferon-γ levels (Mansouri et al. 2017), a potent pro-inflammatory cytokine. Lastly, uraemic patients exhibit reduced γδ T-cell chemokine receptor expression (e.g. CCR5, CXCR3) and poor cytokine secretion profiles (IFN-γ, TNF-α) in response

to a bacterial challenge – these effects are exacerbated in those with latent tuberculosis infection (Juno et al. 2017).

Like T cells, B cells can be indirectly influenced by uraemia via alterations in IL-4 and IL-12 levels which can stimulate or inhibit humoral immunity, respectively (Girndt et al. 2001; Libetta et al. 2001). B cells can also be directly affected by uraemia. B cell lymphopenia has been demonstrated in ESRD in several studies (Bouts et al. 2004; Fernandez-Fresnedo et al. 2000; Pahl et al. 2010). Using an *in vitro* model, Fernandez-Fresnedo et al demonstrated that B cells isolated from uraemic pre-dialysis CKD patients showed increased Fas expression (an apoptotic marker) when compared to healthy controls, as well as reduced Bcl-2 expression, a protein located in the cell membrane responsible for the repression of cell death. B cells from uraemic patients also display reduced activation in response to *Staphylococcus aureus* (SA) when compared to healthy controls (Degiannis et al. 1987). However, as the response to Epstein-Barr virus (a T cell-independent B cell activator) was unchanged, the reduced response to SA (a T cell-dependent B cell activator) is considered to represent defective T cell function. Regardless, B lymphocytes from uraemic patients displayed significantly reduced spontaneous *in vitro* IgA, IgG and IgM release, suggesting a suppression of humoral immune function. There is comparably less research investigating B cells in uraemia than T cells. Interestingly, Vaziri et al (2012) propose that this area of research is limited because the majority of studies have used B cells isolated from blood as opposed to the bone marrow or lymphoid tissues which are important sites for B cell maturation and development.

In summary, uraemia in ESRD leaves the immune system chronically over-activated and as such creates a state of functional anergy, increasing both infection susceptibility and chronic systemic inflammation. This is summarised in Figure 2.2.

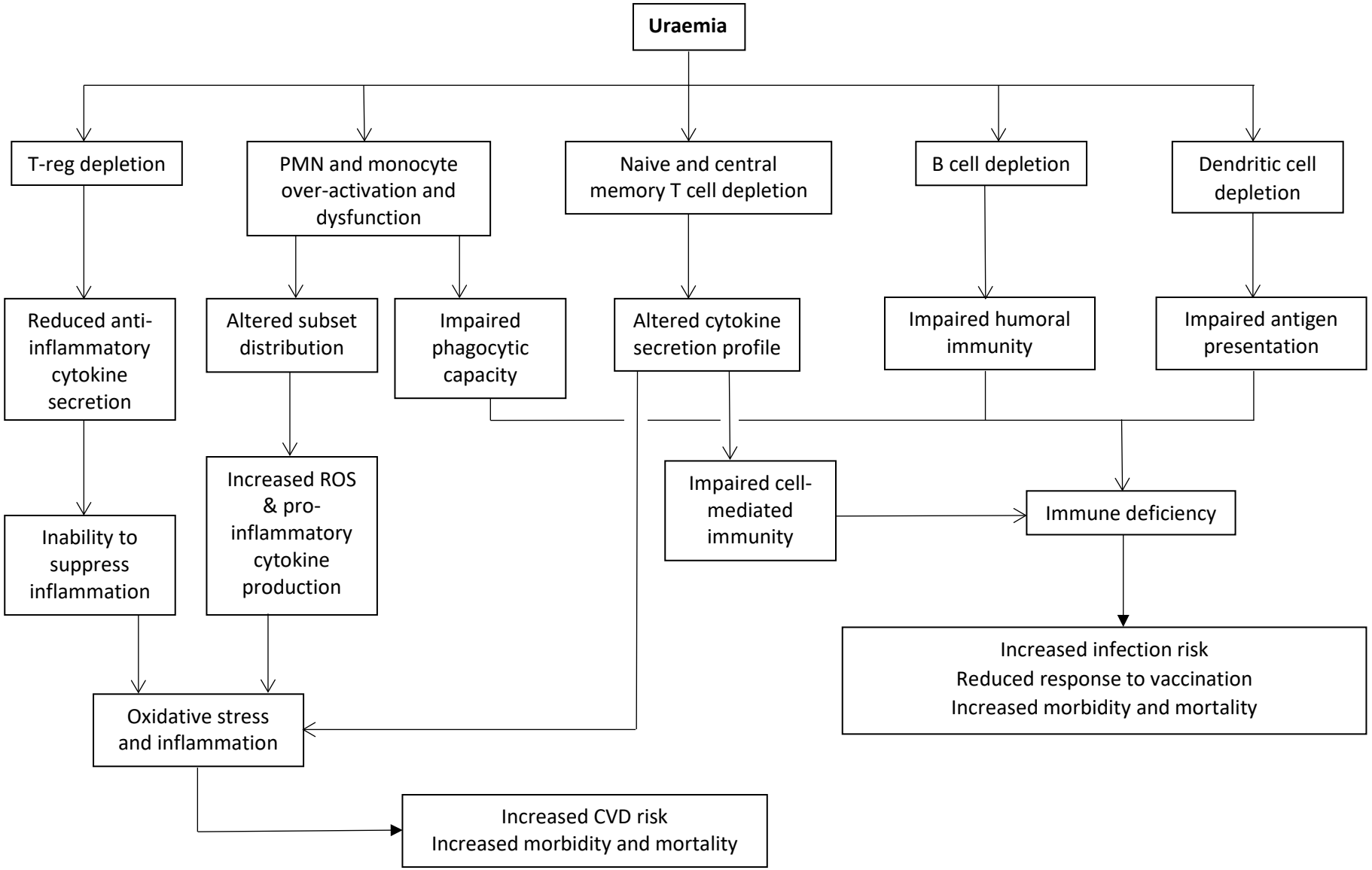

Figure 2.2. Summarising the widespread impact of uraemia on the immune system. Adapted from Vaziri et al (2012).

Haemodialysis

In addition to uraemia, haemodialysis (HD) treatment *per se* can impair immune function and propagate systemic inflammation, primarily due to the contact between the blood, extracorporeal circuit and dialysate which increases endotoxin exposure and causes ligation of immune cell receptors. Acute HD-initiation can cause profound and rapid neutropenia (Toren, Goffinet, and Kaplow 1970), possibly caused by adhesion to the dialysis membrane (Cheung, Hohnholt, and Gilson 1991). HD can also decrease neutrophil phagocytic capacity (Anding et al. 2003; Waterlot et al. 1985; Zein et al. 2015) which may in part be caused by membrane bio-incompatibility (Vanholder et al. 1996). However, some researchers suggest that acute HD can actually increase phagocytic, degranulation and oxidative burst capacity (Anding et al. 2003; Cheung, Parker, and Hohnholt 1993; Gastaldello et al. 2000; Patruta et al. 1998). It may be that HD *per se* can impair neutrophil function, whilst the associated acute reduction in uraemia can reduce the impairments explained above.

HD patients display monocytopenia (Nockher, Wiemer, and Scherberich 2001), a shift towards the intermediate $CD14^{+}CD16^{+}$ subset (Nockher and Scherberich 1998) (Malaponte et al. 2002) and decreased antigen presentation capacity (Agrawal et al. 2009). Monocytes from HD patients also display increased spontaneous but decreased stimulated pro-inflammatory (IL-6, IL-1β and TNF-α) cytokine secretion, suggesting exhaustion and dysfunction caused by chronic activation. These effects may increase with greater HD vintage, and have been associated with increased infection rates (Nockher and Scherberich 1998). HD membranes can also cause aberrant complement activation and thus increased C3 cleavage via the alternative pathway (Hakim, Fearon, and Lazarus 1984), the degree of which may depend on the biocompatibility of the membrane (Deppisch, Göhl, and Smeby 1998). Reduced biocompatibility can also increase LPS-stimulated monocyte IL-6 and IL-1β secretion (Girndt et al. 1999) and reduce natural killer cell cytotoxicity (Zaoui and Hakim 1993) in ESRD patients. Additionally, the C3a and C5a fragments produced during complement activation (and over-activation) can act as ligands for a variety of leukocytes (e.g. monocytes and mast cells) triggering the release of such pro-inflammatory cytokines as IL-6 and TNF-α and the chemokines IL-8 (Nilsson et al. 2007), MCP-1 (Torzewski et al. 1996)

and RANTES (Venkatesha et al. 2005). This would increase the chemotaxis and endothelial adhesion of neutrophils, monocytes and T cells respectively, possibly stimulating atherosclerosis if occurring on a chronic and systemic scale.

HD also affects the adaptive immune system. HD can cause naive and central memory T cell lymphopenia and a reduction in the $CD4^+/CD8^+$ T cell ratio (Yoon et al. 2006), suggesting a more terminally differentiated immune system and thus impaired *de novo* pathogen response. HD patients therefore also display an increased neutrophil-lymphocyte ratio, which has been suggested to be an inflammatory marker and is associated with increased cardiovascular and all-cause mortality (Neuen et al. 2016). HD also reduces PHA-stimulated T cell proliferation and increases CD95 and Annexin-V expression (Moser et al. 2003), suggesting increased rates of apoptosis. Vaccination efficacy is also impaired - Hepatitis B vaccination elicits reduced antibody numbers in HD patients (Peces et al. 1997), which likely explains the elevated incidence of Hepatitis B in this population (Edey, Barraclough, and Johnson 2010). The altered cytokine secretion profile (i.e. increased INF-γ and IL-12) in HD patients also favours Th1 and thus T cell mediated immunity and reduces Th2 B cell mediated humoral immunity (Sester et al. 2000), which may explain the reduction in vaccination efficacy. However, whilst this shift would be expected to reduce B cell antigen response (i.e. production of short-lived, antibody producing plasma cells and long-lived memory cells), it does not explain the reduction of circulating B cells previously observed in HD patients (Bouts et al. 2004). Whilst the cause of both B and T lymphopenia in HD is unclear, possible contributory mechanisms could include reduced lymphopoiesis, increased apoptosis/necrosis and adhesion to the dialyser membrane. The presence of other comorbidities (e.g. diabetes mellitus, malignancy, hepatitis C) can also reduce seroconversion rates in HD patients receiving Hepatitis B vaccinations (Cordova et al. 2017).

Renal Transplantation

Unsurprisingly, the immune system of renal transplant recipients is influenced by the immunosuppressive medication required to prevent allograft rejection. What is surprising, however, is

the apparent dearth of research investigating immune function in this patient population. The research to date is summarised below.

The number and phagocytic capacity (measured using *Staphylococcus Aureus* stimulation) of neutrophils in RTRs has been shown to be similar to that of the healthy population, whilst oxidative burst capacity is reduced (Hutchinson et al. 2003). Similarly to HD patients, RTRs display a shift towards the pro-inflammatory intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocyte subsets 3-6 months post-transplantation when compared to healthy individuals, which results in enhanced production of TNF-α, IFN-γ and IL-1β (Vereyken et al. 2013). However, in RTRs of a greater transplant vintage (6-7 years) a reduction in the percentage of $CD14^{+}CD16^{++}$ has been shown (Boersema et al. 2015). Possible explanations for this effect include prolonged glucocorticoid use (Dayyani et al. 2003) or the increased presence of chronic transplant dysfunction promoting selective monocyte migration into the allograft (Boersema et al. 2015). Little is currently known about monocyte function in RTRs.

Lymphocyte subsets are also altered in RTRs compared to the general population. Decreased percentages of $CD19^{+}CD20^{+}$ B cells, $CD3^{+}CD4^{+}$ T cells, $CD4^{+}CD25^{+}$ regulatory T cells and $CD3^{-}CD16^{+}CD56^{+}$ NK cells and increased percentages of $CD3^{+}CD8^{+}$ T cells have been reported post-transplantation (Calarota et al. 2012; Hutchinson et al. 2003; Wood and Sakaguchi 2003) – these shifts have been positively correlated with opportunistic infection incidence (Calarota et al. 2012). The shift from $CD4^{+}$ T-helper cells to $CD8^{+}$ cytotoxic T cells suggests a more terminally differentiated immune system less able to deal with novel pathogens, whilst the decrease in T-reg percentage may limit graft tolerance (Wood and Sakaguchi 2003). Interestingly, differences in the combination therapy of RTRs may influence the depletion of T-regs, with calcinuerin inhibitors suggested to be more detrimental than rapamycin inhibitors (Segundo et al. 2006).

In addition to their concentrations, lymphocyte function may also be altered. RTRs have displayed a significant reduction in PHA-stimulated lymphocyte proliferation when compared to healthy controls (Hutchinson et al. 2003) - although it is not clear from this study which specific lymphocytes were affected. There is very little research investigating lymphocyte function in RTRs. Given that T-regs are

implicated in the maintenance of graft tolerance (Wood and Sakaguchi 2003) and cytotoxic T cells are implicit in mounting an allo-immune response to the transplanted kidney and promoting graft rejection (Vasconcellos et al. 1998), the lack of research is surprising.

Overall, given the necessity to strike a fine balance between over-suppression (risking recurrent opportunistic infections) and under-suppression (risking graft rejection), there is a discernible lack of literature concerning immune system characteristics and function in RTRs.

2.5 Microparticles

Microparticles (MPs) are shed membrane vesicles, usually ranging in size from 0.1 to 1.0 μm. They are distinct from exosomes, which tend to be smaller (<0.1 μm) and have a different method of formation (Cocucci, Racchetti, and Meldolesi 2009). For a full review regarding MPs and exercise in clinical populations, see Highton et al (2018).

Causes of Formation

MPs are released from the cell membrane during apoptosis or activation, elicited by a variety of stimuli. For example, the activation stimuli could be inflammation, oxidative stress or mechanical/haemodynamic fluctuations depending on the parent cell in question. After their formation, MPs express surface proteins and antigens that are suggestive of their cellular origin, through which they can be identified by laboratory techniques (the most commonly measured MP cellular sources and their corresponding surface antigens are listed in Table 2.5). The MP membrane also includes negatively charged phospholipids, a large proportion of which are phosphatidylserine (PS) which is exposed on the outer layer (Shantsila, Kamphuisen, and Lip 2010).

MP Cellular Source	Surface antigen/s used for determination
All cells	Phosphatidylserine*
Leukocyte	CD11a, CD45
Granulocyte	CD11b, CDF66
Platelet	CD31 (PECAM-1), CD40L, CD41/a, CD42b, CD61
Monocyte	CD11b, CD14, CD16
Endothelial cell	CD31, CD51, CD54 (ICAM-1), CD62E (E-Selectin), CD62P (P-Selectin), CD105 (endoglin), CD144 (VE-Cadherin), CD146 (S-Endo 1)
Neutrophil	CD66b
Erythrocyte	CD235a
Lymphocyte	CD3, CD4, CD36

Table 2.5. The most common cellular sources of MPs, along with the corresponding antigens exposed on their outer surface. As many cellular sources can be represented by several cell surface markers, differences may occur in the literature when studies have used different markers for the same MP derivation, which could lead to inaccuracies. CD = Cluster of Differentiation, PECAM = platelet-endothelial cell adhesion molecule, ICAM = intercellular adhesion molecule. *The value of measuring PS (by assessing the degree of ligation with its detector reagent Annexin-V) to quantify 'all MPs' has been questioned; as many as 80% of MPs do not bind with Annexin-V *in vitro* and therefore do not express PS on their outer surface (Connor et al. 2010). PS-negative MPs which do not bind with Annexin-V demonstrate reduced pro-coagulant activity compared to their PS-positive counterparts (Connor et al. 2010) however their functional significance remains unclear and warrants further investigation. (Highton et al. 2018).

Mechanisms of Formation

A resting, inactivated phospholipid membrane will display phospholipid asymmetry, i.e. different phospholipids displayed on the outer and inner layer (PS is displayed on the inner layer in a healthy membrane (Bevers et al. 1999)). This asymmetry is maintained by cytosolic and transmembrane enzymes such as gelsolin, aminophospholipid translocase, floppase, scramblase and calpain. Cellular activation or apoptosis causes the endoplasmic reticulum to release calcium into the cytosol, which alters the actions of these enzymes, resulting in a restructuring of the cytoskeleton and a reversal of the phospholipid asymmetry and therefore externalisation of PS. This causes outward 'blebbing' of the cell membrane and ultimately fissure, resulting in a released vesicle that might express both PS and surface proteins related to its parental cell on its outer membrane. This process is displayed in Figure 2.3.

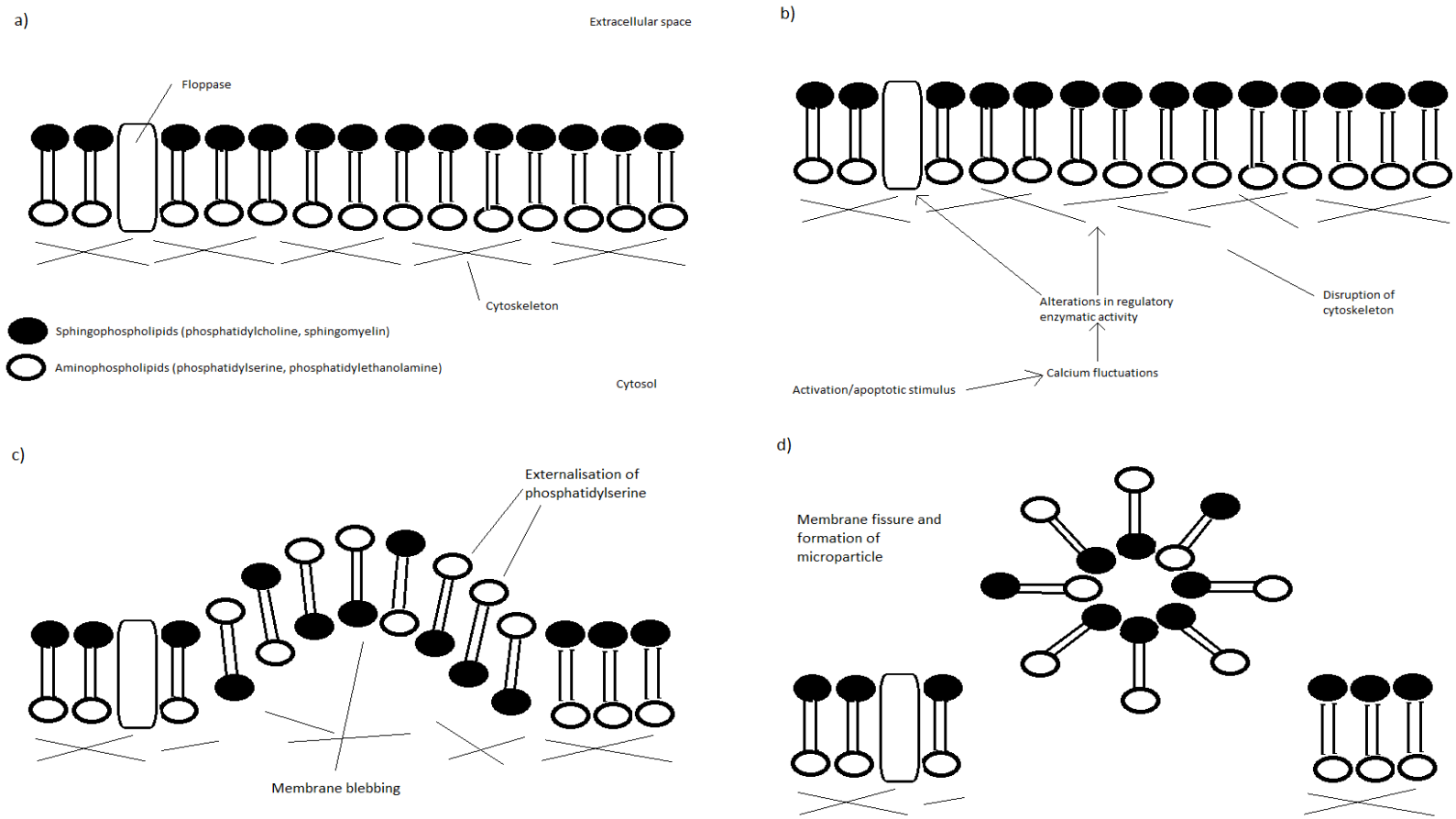

Figure 2.3. The steps involved in the process of MP formation. a) Demonstrating a healthy membrane, with phospholipid asymmetry and the presence of the regulatory enzyme floppase (interchangeable in this diagram with the other regulatory enzymes mentioned). b) Activation or apoptotic stimuli cause fluctuations of cytosolic calcium, altering the activity of the regulatory enzymes and causing cytoskeletal disruption. c) Membrane blebbing and loss of phospholipid asymmetry resulting in externalisation of phosphatidylserine. d) Fissure of the membrane, resulting in the formation of an MP which is now a distinct vesicle from its original membrane. This MP will express surface antigens representative of its parent cell, which can be assessed to identify the origin of the MP. The MP size and number of phospholipids present in the membrane in Figure d) is not truly representative; the purpose of this diagram is to illustrate the formation process. In reality, the MP is of far greater size relative to the phospholipids, which are also present in far greater abundance in the MP membrane. Diagram taken from Highton et al (2018).

Functions

As MPs are released in response to cellular stress, they are elevated in a variety of disease states and can be used as biomarkers of disease severity. MPs are elevated in a number of chronic systemic inflammatory conditions (Distler et al. 2006) including rheumatoid arthritis (Knijff-Dutmer et al. 2002) and systemic lupus erythematosus (Sellam et al. 2009), cardiovascular diseases (Vanwijk et al. 2003) including stroke and acute coronary syndrome patients (Mallat et al. 2000; Simak et al. 2006), various forms of cancer including colon, prostate, breast, ovarian and gastric cancer (Goon et al. 2006; Kanazawa et al. 2003), HIV (Baker et al. 2013), and various forms of renal disease including pre-dialysis chronic kidney disease, patients receiving varying dialysis modalities and renal transplant recipients (Burton et al. 2013; Daniel et al. 2008). Many other conditions have been associated with increased MP levels – their rather unspecific nature of release (i.e. upon an activation or stress stimuli) means that a wide variety of stimuli can elicit MP shedding from a large number of cell types.

More recently, MPs have also been considered as biologically active with effector functions rather than simply biomarkers of disease (Chironi et al. 2009). Endothelial-cell derived MPs can induce endothelial activation and dysfunction (Amabile et al. 2005) by reducing endothelium-dependent vasodilation in response to acetylcholine (Boulanger et al. 2001; Martin et al. 2004) and decreasing the release of the vasodilation-inducing NO (Brodsky et al. 2004; Martin et al. 2004) when incubated *in vitro* with rat aortic rings. This can reduce the ability of the vasculature to respond to fluctuations in haemodynamic pressure, inducing cardiac stress and left ventricular hypertrophy (Treasure et al. 1993), and increasing cardiovascular mortality (Bauml and Underwood 2010). Similarly, angiotensin II, which promotes vasoconstriction via activation of the renin angiotensin system and thus increases cardiovascular risk (Schmieder et al. 2007) can increase endothelial-cell derived MP release when incubated *in vitro* with murine endothelial cells (Burger et al. 2011), indicating endothelial damage. Increased circulating count of MPs of many cellular derivations have been positively associated with the circulating concentration of several reactive oxygen species (ROS), including plasma glutathione peroxidase and superoxide (Helal et al. 2011; Mastronardi et al. 2011). When endothelial-cell derived MPs are incubated *in vitro* with

human umbilical vein endothelial cells (HUVECs), the detrimental changes seen in angiogenesis (e.g. a reduction in total capillary length) were alleviated the presence of superoxide dismutase (Mezentsev et al. 2005), implicating ROS production as a potential mechanism by which MPs can impair vascular function.

Endothelial-cell derived MPs released from HUVECs in response to the pro-inflammatory cytokine TNF-α have a high calcium content and can induce osteogenesis and calcification when incubated with vascular smooth muscle cells (Buendia et al. 2015). Similarly, platelet-derived MPs incubated with rat aortic rings can promote angiogenesis via increased vascular endothelial growth factor (VEGF) activity (Brill et al. 2005) whilst endothelial cell-derived MPs incubated with HUVECs can increase PI3K activity, which plays a critical role in angiogenesis (Deregibus et al. 2007). Whilst angiogenesis is important for maintaining vascular health and homeostasis (Moreno, Sanz, and Fuster 2009), excessive or dysregulated angiogenesis has been implicated in many conditions, including cancer (via loss of tumour growth suppression), some autoimmune disorders, atherosclerosis, pulmonary hypertension and inflammatory bowel disease, among others (Carmeliet 2003). These effects on the vasculature may increase cardiovascular risk and thus risk of mortality (Nevskaya et al. 2008; Siervo et al. 2010). Lastly, PS externalised on MPs can bind with the pro-thrombotic and pro-coagulant tissue factor (TF) to initiate and promote thrombosis and coagulation (Ando et al. 2002; Polgar, Matuskova, and Wagner 2005; Sinauridze et al. 2007) increasing the risk of embolism and driving atherosclerosis (Kleinegris, Ten Cate-Hoek, and Ten Cate 2012). Elevated MP counts might therefore be predictive of mortality in a variety of conditions (Amabile et al. 2012; Bharthuar et al. 2013; Wang et al. 2014).

Microparticles in ESRD

There is a small but growing body of research concerning MPs within the context of CKD and ESRD. Platelet, endothelial cell and lymphocyte-derived MPs are elevated compared to the general population in both pre-RRT CKD patients (Chen et al. 2014; Faure et al. 2006; Lu et al. 2015; Rodrigues et al. 2018) and HD patients (Burton et al. 2013; Trappenburg et al. 2012) – this effect may be greater in patients with concurrent diabetes mellitus (Carmona et al. 2017; Rodrigues et al. 2018). It has been suggested

that uraemic toxins (e.g. p-cresol, indoxyl sulfate) can directly promote the shedding of MPs from parent cells (largely EMPs) (Carmona et al. 2017; Meijers et al. 2009). Whilst the mechanism behind this relationship is unclear, increased toxin-stimulated ROS production causing endothelial activation and toxin-stimulated vascular smooth muscle cell dysfunction have been implicated (Carmona et al. 2017; Henaut et al. 2018). The HD process *per se* may also increase circulating platelet neutrophil-derived MPs over a single dialysis session (Daniel et al. 2006) – it has been suggested that the inflammatory stimuli associated with the HD process causes increased parent cell activation and hence MP shedding.

Elevated MP counts in ESRD patients have been associated with increased thrombus generation (Burton et al. 2013) and hence increased risk of thrombotic events (e.g. stroke) (Ando et al. 2002), increased endothelial damage (Carmona et al. 2017) and dysfunction (Amabile et al. 2005), reduced endothelial progenitor cell count and increased vascular calcification risk (Soriano et al. 2014) and ultimately increased cardiovascular mortality (Amabile et al. 2012; Chen et al. 2014). Interestingly, following successful renal transplantation, lymphocyte, platelet and neutrophil-derived MP counts are decreased at 3 months post-transplantation and further reduced at 9 and 12 months post. The MP pro-coagulant activity is also decreased following renal transplantation, however TF expression remains unchanged (Al-massarani et al. 2009).

Whilst the body of research concerning MPs in clinical populations is growing, many issues remain that make drawing meaningful conclusions difficult. These include the large proportion of *in vitro* studies, the small sample size of the majority of human studies (likely due to the cost and labour-intensive nature of assessing and quantifying MPs) and the lack of uniformity in the methodology implemented to isolate and assess MPs (Erdbrugger and Le 2016).

There are several different laboratory techniques that are regularly used for the identification of circulating MPs in the literature. Broadly, these include: flow cytometry; transmission electron microscopy; nanoparticle tracking analysis (NTA), and resistive pulse sensing. Whilst flow cytometry generally has a higher minimum detectable threshold than other techniques and can be time and labour intensive, it provides the most information with regards to MP size, complexity and cellular surface

marker expression, and therefore remains the 'gold standard' technique most applicable to clinical research (van der Pol et al. 2014; Strasser et al. 2013). Flow cytometry also provides high throughput whilst remaining relatively cheap, making it desirable when compared to other techniques (Szatanek et al. 2017). Sample collection and preparation techniques, including needle gauge and anticoagulant used for sample collection, tourniquet use, centrifugation protocol, freezing and thawing protocol, and buffer filtration may also influence the detection of total (Dey-Hazra et al. 2010; van der Heyde et al. 2011) and phenotype-specific MPs (van Ierssel et al. 2010).

2.6 Physical activity and exercise

The beneficial effects of participating in regular physical activity and/or structured exercise training are well documented. Low levels of physical activity and the consequential reduced physical fitness are associated with an increase in both all-cause and cardiovascular mortality in the general population (Lee et al. 2012). In England, over one in four women and one in five men are classified as 'inactive' – i.e. they do less than 30 minutes of physical activity a week (this number increases with age - roughly half of all adults aged over 75 are classified as inactive) (Scholes 2017). The increase in morbidity and mortality as a result of physical inactivity places a major strain on healthcare systems both in the UK (Scarborough et al. 2011) and globally (Oldridge 2008). Physical inactivity has been reported as the fourth largest cause of disease and disability in the UK (behind tobacco smoking, hypertension and obesity) (Murray et al. 2013). As such, Public Health England suggests physical inactivity is responsible for roughly one in every six deaths (Lee et al. 2012), and costs the UK an estimated £7.4 billion a year (Scarborough et al. 2011).

Effects of physical activity and exercise on inflammation in the healthy population

The evidence supporting the systemic beneficial effects of exercise in the general population is both overwhelming and comprehensive. Large observational studies have suggested that engaging in regular physical activity, particularly of an aerobic nature, is protective against CVD (Thompson et al. 2003), type II diabetes mellitus (Sigal et al. 2006), various forms of cancer (Kyu et al. 2016), chronic obstructive

pulmonary disease (Spruit et al. 2015) and cognitive decline resulting in dementia (Blondell, Hammersley-Mather, and Veerman 2014).

Regular physical activity has also been reported to be a potent anti-inflammatory stimulus in the general population. Retrospective analysis of two large study cohorts (n = 5,888 and n = 3,638) found that increased habitual physical activity levels were associated with a decrease in a battery of pro-inflammatory markers (e.g. CRP, white blood cell count, fibrinogen) in the healthy population across varying ages (Abramson and Vaccarino 2002; Geffken et al. 2001). The proposed mechanisms behind the anti-inflammatory stimulus of exercise are numerous and include: release of IL-6 from the contracting muscle, initiating an anti-inflammatory cascade characterised by increased IL-10 and IL-1ra levels resulting in a downregulation of major histocompatibility complex (MHC), adhesion molecule and costimulatory molecule (CD80 and CD86) expression on antigen presenting cells and a reduced effector T cell secretion of pro-inflammatory cytokines; activation of the hypothalamic-pituitary-adrenal axis resulting in increased levels of circulating cortisol and adrenaline which suppress pro-inflammatory cytokine (e.g. TNF-α and IL-1β) production; inhibition of macrophage infiltration into adipose tissue and promotion of the phenotype-switching from M1 to M2 macrophages, which preferentially produce anti-inflammatory mediators; down-regulation of toll-like receptor (TLR) expression on monocytes, resulting in reduced monocyte activation and pro-inflammatory cytokine secretion; reduced proportion of inflammatory ($CD16^+$) monocytes in the circulation, resulting in reduced monocyte TNF-α secretion; and increased circulating numbers of T-regs, resulting in increased anti-inflammatory cytokine (IL-10, TGF-β) production (Gleeson et al. 2011). Regular physical activity can also indirectly influence inflammation by eliciting changes in body composition. Visceral adipose tissue is a source of pro-inflammatory adipokines such as TNF-α, leptin, IL-6, IL-8 and MCP-1, whilst skeletal muscle can induce an anti-inflammatory environment as explained earlier, so the favourable changes in body composition that accompany regular exercise (i.e. reduced adipose tissue mass, increased skeletal muscle mass) exerts an overall anti-inflammatory influence (Gleeson et al. 2011). The relative contribution of each of the above mechanism towards the overall anti-inflammatory effect of exercise is unknown and likely depends on the individual completing the exercise. For instance, an overweight or obese individual is likely to see

anti-inflammatory effects of exercise as a result of a decreased pool of adipose tissue-resident macrophages than an already lean or trained individual. Similarly, a resistance trained individual may see a greater anti-inflammatory response as a result of skeletal muscle IL-6 secretion than a non-resistance trained individual due to their increased skeletal muscle mass. The type, duration and intensity of the exercise may also influence the relative contribution of each mechanism. For instance, prolonged exercise may exert an anti-inflammatory effect largely via skeletal muscle IL-6 release due to glycogen depletion (Keller et al. 2005). However, it is likely that all the above mechanisms contribute to some degree towards an anti-inflammatory environment, regardless of the individual or exercise in question.

Regular exercise also positively impacts the immune system in the general population. The 'J-shaped curve' relationship between exercise volume (frequency and intensity) and upper respiratory tract infection (URTI) suggests that moderate amounts of exercise reduces infection risk, whilst no exercise (sedentary) and high volumes of exercise increase infection risk (Nieman 1994), suggesting that regular moderate intensity exercise bolsters immune function. This relationship is displayed in Figure 2.4.

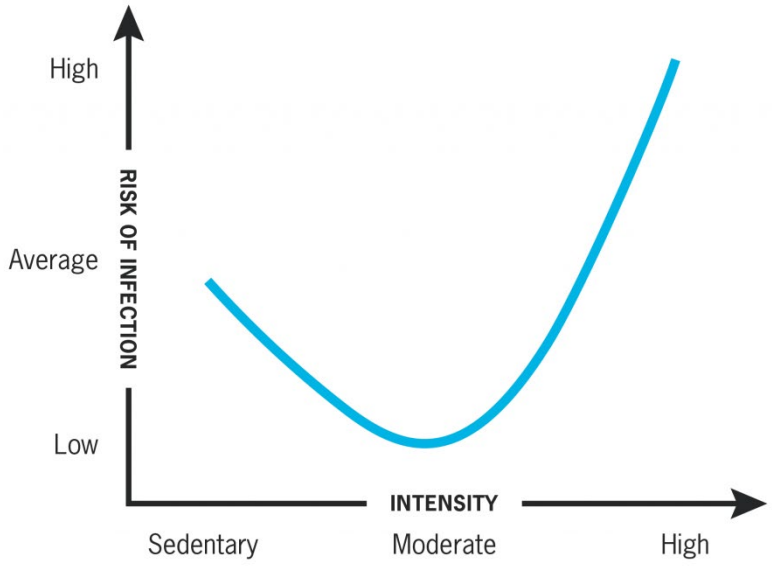

Figure 2.4. The J-shaped curve of exercise volume and URTI risk (Nieman 1994).

On an acute basis, exercise, particularly of an aerobic nature, tends to suppress immune function and creates the post-exercise 'open window' for infection risk (Nieman and Pedersen 1999), whereby

infection risk is transiently increased post-exercise (3 to 72 hours depending on the exercise implemented and immune parameter measured). Briefly, this is due to a reduction in monocyte and lymphocyte IL-2 and IFN-γ production, increased IL-6 production by skeletal muscle, increased catecholamine (epinephrine and cortisol) release from the adrenal and pituitary glands, and increased plasma concentrations of IL-10 and IL-1ra. All of these changes result in a shift away from Type 1 T cell mediated immunity and towards Type 2 T cell and humoral immunity, thus decreasing virus protection capacity and increasing URTI risk (Gleeson 2007). These effects are enhanced with increasing exercise intensity and duration.

Conversely, and as seen in Figure 2.4, regular moderate intensity exercise strengthens resting immune function, provided adequate recovery time is implemented between sessions. The suggested mechanisms behind this relationship include: lower inflammatory monocyte counts and stimulated pro-inflammatory cytokine secretion; increased dendritic cell number and MHC II expression; and increases in natural killer cell count and cytotoxicity (although this is debated as conflicting results have been found) (Walsh et al. 2011).

Additionally, acute aerobic exercise has exhibited the ability to influence MP numbers in healthy participants. Aerobic exercise elicits increased blood flow to meet the extra oxygen demands of the working muscles, which can modify haemodynamic activation of both freely circulating cells and cells adhered to the endothelium via alterations in shear stress. Shear stress is a product of blood viscosity and flow rate; therefore aerobic exercise-induced increased blood flow can increase shear stress (Long et al. 2004), which has been implicated in MP formation and release via modulation of cell membrane quiescence (Kim et al. 2015; Miyazaki et al. 1996; Reininger et al. 2006) due to haemodynamic cellular activation. Acute aerobic exercise may also increase cellular activation by transiently increasing catecholamine (e.g. norepinephrine) levels (Perini et al. 1989), thus increasing MP shedding by lowering membrane quiescence. Lastly, acute aerobic exercise can increase leukocyte apoptosis (Mooren, Lechtermann, and Völker 2004), potentially triggered by increases in cellular oxidative stress caused by

increased reactive oxygen species production (Phaneuf and Leeuwenburgh 2001). As MPs are released by apoptotic cells (Distler et al. 2005), this exercise-induced apoptosis also increases MP production.

However, the findings seem to be conflicting; some studies report increased post-exercise MP counts of platelet origin, particularly after strenuous exercise (Chaar et al. 2011; Chen et al. 2015; Chen, Chen, and Wang 2010; Chen, Chen, and Wang 2013; Sossdorf et al. 2010), which suggests a pro-thrombotic effect due to the high TF expression usually found on platelet-derived MPs (Del Conde et al. 2005; Diamant et al. 2002). Mechanical activation of platelets and thus accelerated MP shedding is cited as the cause of this. Conversely, other studies have found no change in endothelial cell or platelet-derived MP levels following high-intensity (100% peak power output) cycling (Guiraud et al. 2013) or even observed a reduction in circulating EMPs following cycling of various intensities (55-100% peak power output) (Wahl et al. 2014). This disparity may be caused by training status; the studies mentioned above which found increased MPs used healthy but sedentary (i.e. exercise frequency of ≤ 1/week) participants, whilst those that found decreased MPs used trained participants (either author-defined as 'fit' (Guiraud et al. 2013) or trained triathletes and cyclists (Wahl et al. 2014)). This hypothesis is supported by studies investigating chronic regular aerobic exercise training, which display both an attenuation in the acute exercise-induced increase in MPs (neutrophil and platelet derived) (Chen et al. 2015; Chen et al. 2013) and a reduction in resting EMP counts (Babbitt et al. 2013; Kim et al. 2015) following prolonged training (e.g. 3 times/week for 6 months). Therefore, in the general population, it seems that whilst acute aerobic exercise may increase circulating MP counts, regular aerobic exercise training can either attenuate or abolish this effect and reduce resting circulating MP levels. This may be due to an adaptation effect caused by the repeated exposure of the endothelium to high shear stress elicited by aerobic exercise, which would prevent endothelial leukocyte adhesion and endothelial cell activation and/or apoptosis. Regular exercise training also improves endothelial function and increases resting NO availability (Green et al. 2004), which may partially mitigate the increased shear stress caused by increased blood flow and thus prevent MP formation as explained above (Mezentsev et al. 2005).

Effects of physical activity and exercise in the CKD and ESRD populations

Many of the beneficial systemic effects of regular physical activity and exercise seen in the general population are also mimicked in the CKD and ESRD populations. Comprehensive systematic reviews and meta-analyses have shown that regular exercise training, regardless of modality, has been shown to increase aerobic capacity, increase muscular strength, improve body composition, increase heart rate variability and increase health-related quality of life in both CKD and HD patients (Heiwe and Jacobson 2014; Smart and Steele 2011). As such, peak VO_2 is a powerful predictor of mortality in the ESRD population, even more so than age, dialysis vintage or diabetes and heart failure presence (Sietsema et al. 2004). Similarly, increased habitual physical activity levels have been linked with reduced mortality in ESRD patients (Zhang et al. 2017).

Whilst the beneficial effects of regular, moderate intensity aerobic exercise on systemic inflammation and the immune system are well documented in the general population (Gleeson et al. 2011; Nieman and Pedersen 1999; Walsh et al. 2011), there is less research within the context of CKD and ESRD. A comprehensive list of the studies that have investigated the impact of acute exercise and regular exercise training on immune function and inflammation in pre-RRT CKD patients is included in Table 2.6, whilst those that have investigated these relationships in ESRD are included in Table 2.7. In pre-dialysis CKD patients, 6-months of moderate intensity (RPE 12-14) walking training (30 min/d, 5 times/wk) reduced plasma IL-6 levels and increased plasma IL-10 levels (Viana et al. 2014). $CD4^+$ and $CD8^+$ T cell CD69 expression and $CD14^+$ monocyte CD86 expression (markers of activation) were also reduced, whilst total and differential leukocyte count and neutrophil degranulation (bacterially-stimulated elastase concentration) remained unaffected. Conversely, other studies that have investigated regular, moderate intensity aerobic exercise (3x/wk, 24-48 wks) in pre-dialysis CKD patients have displayed no change in CRP (Headley et al. 2012; Leehey et al. 2009), although a more recent study found that whilst a single 20 min bout of moderate intensity (50% VO_2peak) cycling in paediatric CKD patients did not impact circulating IL-6 or TNF-α concentrations, there was an increase in the number of circulating NK^{bright} cells (natural killer cells) (Lau et al. 2015), suggesting the ability to deal with novel virally infected

Study	Acute exercise/Regular exercise training	Sample size and study design	Intervention	Results (concerning inflammation)
Castaneda et al. 2004 (Castaneda et al. 2004)	Regular exercise training	RCT (14 exercise, 12 control)	Resistance exercise, 3 sets of 8 reps at 80% 1 RM, 5 machines (3x/wk, 3 months)	CRP decreased by 1.7 mg/L in exercise group but increased by 1.5 mg/L in control group IL-6 decreased by 4.2 pg/mL in exercise group but increased by 2.3 pg/mL in control group
Headley et al. 2012	Regular exercise training	RCT (10 exercise, 11 control)	Aerobic exercise 50-60% VO2peak (55 min, 3x/wk, 48wks)	No change in CRP or IL-6
Lau et al. 2015 (Lau et al. 2015)	Acute exercise	Uncontrolled (n = 9, paediatric)	Cycling 50% VO_{2peak} (20 min)	Increase in NK^{bright} cells (7.4 ± 4.3 vs. 12.2 ± 8.3×10^6 cells/L; p=0.02) No change in IL-6 or TNF-α
Leehey et al. 2009	Regular exercise training	RCT (7 exercise, 4 control)	Waling exercise (30 min, 3x/wk, 24 wks)	No change in CRP
Van Craenenbroeck et al. 2014	Acute exercise	Disease-controlled (20 CHF, 20 CKD, 15 healthy control)	Maximal cardiopulmonary cycling exercise test (~10 min)	Exercise increased intermediate and non-classical monocyte % and reduced classical monocyte % in CKD ad healthy control (p < .05) No change in IL-6
Viana et al. 2014	Acute exercise	Uncontrolled (n = 15)	30 min treadmill walking exercise RPE 12-14	Exercise increased IL-6 (pre 7.7 ± 4.7 to post 9.4 ± 6.4 and 1h-post 8.9 ± 5.5 pg/mL, p < .001) and IL-10 (pre 3.2 ± 0.8 to post 3.8 ± 1.1 and 1h-

				post 4.3 ± 1.1 pg/mL, p < .001) No change in CRP Exercise improved bacterially-stimulated per-neutrophil elastase release (pre 510 ± 180 to 1h-post 598 ± 245 fg/cell, p = .003)
Viana et al. 2014	Regular exercise training	RCT (13 exercise, 11 control	Home-based walking RPE 12-14 (30 min, 5x/wk, 6 months)	Resting IL-6/IL-10 ratio reduced in exercisers (p = .001) but not controls (p = .117) Exercise reduced markers of lymphocyte (CD69) (p ≤ .002) and monocyte (CD86, HLA-DR) activation (p ≤ .01) No change in immune cell numbers or neutrophil degranulation

Table 2.6. The studies that have investigated inflammation and immune function in pre-RRT CKD patients. (only studies including human participants are listed). To the author's knowledge, no investigations regarding exercise and MPs have been completed in this population).

Study	Acute exercise/Regular exercise training	Sample size and study design	Intervention	Results (concerning inflammation)
Afshar et al. 2010	Regular exercise training	RCT (7 aerobic training, 7 resistance training, 7 control)	Aerobic = IDC, RPE 12-14 (30 min, 3x/week, 8wks Resistance = RPE 15-17, (3x/wk, 8wks)	Hs-CRP: -83.9% in aerobic, -67.9% in resistance, + 1.5% in control
Afshar et al. 2011	Regular exercise training	RCT (14 exercise, 14 control)	IDC, RPE 12-14 (30 min, 3x/wk, 8 wks)	CRP: -83.2% in exercise, +1.2% in control
Cheema et al. 2011	Regular exercise training	RCT (24 exercise, 25 control)	Intradialytic resistance training (free weights), RPE 15-17 (3x/wk, 12 wks)	No change in TNF-α, IL-1β, IL-6, IL-10 or IL-12 Log CRP: -0.08 in exercise, +0.24 in controls
Daniilidis et al. 2004	Regular exercise training	RCT (20 exercise, 14 control)	Interdialytic aerobic interval exercises, 75-85% peak HR, (60 min, 3x/wk, 6 months)	No change in IL-2, IL-4, IL-6 or T-lymphocyte subsets
Dungey et al. 2015	Acute exercise	Randomised crossover (n = 15)	IDC RPE 12-14 (30 min)	No change in IL-6, TNF-α or IL-1ra
Dungey et al. 2017	Regular exercise training	Non-randomised controlled trial (dialysis unit determined condition) (22 exercise, 16 control)	IDC RPE 12-14 (30 min, 3x/wk, 6 months)	No change in IL-6, TNF-α or CRP. Reduction in % of intermediate monocytes in exercise group only ($p < .001$)
Esgalhado et al. 2015 [Esgalhado et al. 2015]	Acute exercise	Randomised crossover (n = 16)	Lower limb resistance training, 4 exercises, 3 sets of 10 reps 60% 1RM	No change in CRP
Golebiowski et al. 2012	Regular exercise training	Uncontrolled, 29 exercise	IDC (3x/wk, 3 months)	No change in CRP or IL-6
Kopple et al. 2007 [Kopple et al. 2007]	Regular exercise training	RCT (10 endurance exercise, 15 strength exercise, 12 combined exercise, 14 control)	Endurance = IDC 50% VO_{2peak} (3x/wk, 18wks) Strength = interdialytic leg resistance exercise, 3 sets of 8 reps 80% 5 RM (3x/wk, 18 wks)	No change in CRP, TNF-α or IL-6

			Combined = half of endurance, half of strength	
Liao et al. 2016	Regular exercise training	RCT (20 exercise, 20 control)	IDC RPE 12-15 (30 min, 3x/wk, 3 months)	Reduction in CRP (1.25 ± 2.01 to 0.78 ± 0.83 mg/dL, p < .05) and IL-6 (4.23 ± 2.65 to 3.48 ± 2.95 pg/mL, p < .05) in exercise group only
Martin et al. 2018 [Martin et al. 2018]	Acute exercise	Randomised crossover (n = 11)	IDC RPE 12-14 30 min	No exercise-dependent change in MP number or derivation MPs collected 1-hr post-exercise elicited increased ROS response from cultured endothelial cells than control trial
Moraes et al. 2014	Regular exercise training	Uncontrolled (37 exercise)	Intradialytic lower limb resistance band exercise, 4 sets of 10 reps 60% 1 RM (3x/wk, 6 months)	No change in IL-6 or TNF-α Reduction in CRP (2.3 ± 0.9 to 1.6 ± 0.6 pg/mL, p < .001)
Nindl et al. 2004 [Nindl et al. 2004]	Regular exercise training	Uncontrolled (10 exercise)	Interdialytic resistance training, 1-3 sets 15 reps (2x/wk/ 12 wks)	Reduction in CRP from 10.37 ± 2.71 mg/L in week 0 to 6.12 ± 1.07 mg/L in week 12
Peres et al. 2015	Acute exercise	Randomised crossover (n = 9)	IDC RPE 6-7 (modified Borg scale), 20 min	No change in IL-6 or TNF-α, IFN-γ increased in control trial only (p = .048). IL-10 increased in exercise trial only (p = .018)
Toussaint et al. 2008	Regular exercise training	Randomised crossover (n = 10)	IDC (30 in, 3x/wk, 3 months). 1 month washout	No change in CRP
Wilund et al. 2010	Regular exercise training	RCT (8 exercise, 9 control)	IDC RPE-12-14, (45 min, 3x/wk, 4 months)	No change in IL-6 or CRP
Wong et al. 2017	Acute exercise	Randomised crossover (n = 10)	IDC RPE 11-13 (30 min)	IL-6 (p = .047), hs-CRP (p = .036) and TNF-α (p = .005)

				increased following the control trial only
Zaluska et al. 2002	Regular exercise training	Uncontrolled (10 exercise)	IDC (30 min, 3x/wk/ 6 months)	Sig. decrease in CRP (p = .046)

Table 2.7. The studies that have investigated inflammation and immune function in HD patients (only studies including human participants are listed).

cells was improved. Additionally, in pre-dialysis CKD patients, regular resistance exercise training (3x/wk, 3 months) has been shown to reduce resting circulating concentrations of IL-6 and CRP, with increases in these markers seen in the no-exercise control group (Castaneda et al. 2004). In ESRD patients undergoing haemodialysis, a 6-month exercise intervention comprised of 60 minutes of aerobic exercise (steps, treadmill, gymnastics, swimming completed at 75-85% peak heart rate), 3 times a week on non-dialysis days had little effect on serum immunoglobulin levels (IgA, IgM, IgG), complement components (C3, C4), cytokine levels (IL-2, IL-4, IL-6), or lymphocyte subset distribution. However, URTI rates were reduced compared to the no-exercise control group (incidence in 31% of exercisers vs 58% of controls) (Daniilidis et al. 2004), suggesting some improvement in immune function.

Intradialytic cycling (IDC) is implemented during dialysis treatment – this training modality has been shown to improve exercise capacity, increase muscular strength, increase functional capacity, increase dialysis efficacy via improvements in peripheral blood perfusion, increase amino acid uptake and prevent muscle wasting (Singh et al. 2005), whilst circumventing may of the perceived barriers to exercise in the HD population (Delgado and Johansen 2012). Additionally, whilst the physiological adaptations to training may not be as great in IDC as those obtained via more traditional exercise training completed outside of dialysis, IDC provides greater adherence and compliance rates as the patients are not required to sacrifice any more time from their daily routine (Singh et al. 2005). Twenty minutes of moderate intensity IDC prevented the post-HD increase in IFN-γ, slightly reduced post-HD TNF-α concentration and elicited an increase in IL-10 concentration, albeit when tested on a small sample size (randomised crossover trial in 9 HD patients) (Peres et al. 2015). Another, similarly deigned randomised crossover trial (n = 10) found that 30 minutes of IDC did not impact the circulating concentrations of IL-6, TNF-α and CRP, however these markers were increased following the control trial (Wong et al. 2017). This relative reduction in inflammatory mediators may partially offset the pro-inflammatory stimulus of HD (Bitla et al. 2010) and thus prevent the associated endothelial dysfunction and consequential vascular dysfunction. Additionally, preliminary evidence suggests that acute moderate intensity intradialytic cycling (IDC) does not influence monocyte subset distribution or neutrophil function (Dungey et al. 2015). However, 6-months of IDC (thrice weekly) can reduce the

proportion of the pro-inflammatory intermediate monocyte ($CD14^{+}CD16^{+}$) subset and increase the total number of $CD4^{+}CD25^{+}CD127^{-}$ regulatory T cells when compared to non-exercising control patients. This would be expected to reduce systemic inflammation, though no differences were found in IL-6, TNF-α or CRP (Dungey et al. 2017). However, this requires further investigation due to the relatively small sample size and lack of pre-dialysis blood sample preventing the comparison to a true baseline, i.e. without the impact of dialysis which can be a significant pro-inflammatory stimuli (Amore and Coppo 2002). Conversely, other studies investigating regular moderate intensity IDC have demonstrated reductions in resting CRP levels (Afshar et al. 2010, 2011; Zaluska et al. 2002). However, these studies also employ limited samples sizes (Afshar et al. 2010) or include no control condition (Zaluska et al. 2002). Additionally, the impact of intradialytic resistance training on inflammation has been investigated. This exercise modality can be more problematic, given that the HD patients must remain in their dialysis bed/chair and must keep their upper body relatively still in order to not disrupt their vascular access (if they have a brachial AVF or chest catheter). Therefore, intradialytic resistance training is usually accomplished using resistance bands and implemented in the lower limbs only. Whilst an acute bout of intradialytic resistance training may not impact circulating CRP levels (Esgalhado et al. 2015), some studies have shown that regular intradialytic resistance training can lower CRP concentration (Afshar et al. 2010; Headley et al. 2002; Moraes et al. 2014). However, other studies investigating regular aerobic training, resistance training or concurrent training in dialysis patients have demonstrated no impact on circulating markers of inflammation such as IL-6 and CRP (Cheema et al. 2011; Gołębiowski et al. 2012; Kopple et al. 2007; Toussaint, Polkinghorne, and Kerr 2008; Wilund et al. 2010), though some have been uncontrolled (Gołębiowski et al. 2012) or employed small sample sizes (Toussaint et al. 2008; Wilund et al. 2010). However, it is unclear why those with control conditions and larger sample sizes (Cheema et al. 2011; Kopple et al. 2007) did not display significant changes in inflammatory markers.

Another exercise modality that may provide opportunities for HD patients to increase their exercise participation is electrical muscle stimulation (EMS). In EMS, electrodes placed on the skin deliver low-intensity electrical impulses to a specifically targeted muscle, which reacts by contracting as it would in response to a centrally-driven stimulus (Heidland et al. 2013). Therefore, EMS can be used in place of

dynamic exercise in those who are unable to exercise volitionally, for instance due to musculoskeletal impairments or extreme frailty. The duration and intensity of stimulation can be altered to closely represent the standard progressive overload seen in traditional exercise interventions.

Evidence suggests that EMS can be used to prevent intra- and post-HD hypotension, as well as potentially increase dialysis efficacy via increased toxin removal (Farese et al. 2008). EMS can also mimic exercise more closely by increasing muscular strength and functional capacity in HD patients (Roxo et al. 2016; Simo et al. 2015), though has been shown to have no effect on endothelial function as assessed by brachial artery flow-mediated dilation (Schardong et al. 2017). However, there is very little evidence investigating how LF-EMS affects systemic inflammation HD patients. Brüggemann et al investigated 'high' (50Hz, 72.90mA) and 'low' (5Hz, 13.85mA) intensity LF-EMS in HD patients (1 hour/HD treatment, 3 times/week for 4 weeks). They found that whilst both groups improved to a similar degree in quadriceps strength and 6 minute-walk distance, TNF-α was unchanged and IL-10 actually increased in the high intensity group (Brüggemann et al. 2017). Further research should be undertaken which investigates the impact of EMS on systemic inflammation in HD patients, with a particular focus on comparing this modality to the more traditionally undertaken IDC exercise modality.

Finally, only one study has investigated the impact of exercise on MP phenotypes in the ESRD population (Martin et al. 2018), and found that, whilst there were no exercise-dependent changes in MP numbers or phenotypes following 30 minutes of moderate intensity cycling, MP samples collected 1 hour after exercise induced a greater ROS response from cultured endothelial cells than those collected following the control trial. As acute post-exercise pro-inflammatory stimuli are implicated in creating an overall anti-inflammatory environment, the authors suggest this represents an anti-inflammatory effect. Given the ability of MPs to exert a wide variety of pathophysiological influences, particularly with regards inflammation and thrombosis, there is a need to investigate whether MPs can mediate an anti-inflammatory response to exercise in HD patients.

Given that exercise, both inter and intradialytic, can positively impact body composition (Johansen et al. 2006), functional capacity (Storer et al. 2005), quality of life (Wu et al. 2014), and cardiovascular health

(Deligiannis et al. 1999), the dearth of research regarding inflammation and immune function is surprising. As in the general population, moderate intensity exercise training in ESRD patients would be expected to enhance immune function and thus reduce infection rates, reduce systemic inflammation, and ameliorate the detriments caused by obesity and muscle wasting. Further research is necessary to examine the effect of exercise on markers of systemic inflammation and immune function in ESRD.

2.7 Current limitations in the literature

A number of limitations still exist within the current body of literature which make drawing meaningful conclusions difficult. General problems found in many research topics are present, including small sample sizes, inadequately selected or insufficient control conditions and the propensity towards pilot or feasibility studies (Dungey et al, 2013). However, specific limitations and gaps that exist in the literature and that are addressed in the studies included in this thesis include:

- No attempt to investigate the effect of acute exercise on MP TF kinetics;
- A small number of limited trials (either small sample sizes, lack of appropriate baseline samples or inappropriate or non-existent control conditions) investigating the effect of IDC on inflammatory markers and only one investigating MPs;
- Little research concerning the impact of less traditional exercise modalities (i.e. electrical muscle stimulation) on the inflammatory environment in HD patients;
- An almost complete lack of research concerning exercise, inflammation and immune function in renal transplant recipients;
- Measurements of single (or few) inflammatory markers reported in results – whilst the change (or lack thereof) in one single marker may be informative, many pro- and anti-inflammatory mediators contribute to the balance of systemic inflammation and thus a panel of markers would be more valuable.

When regarding the evidence presented in this chapter, it is clear that ESRD patients are a poorly represented population with regards to research concerning exercise, MPs, inflammation and immune

function. Rectifying the above limitations would add to the current body of knowledge and aid in the future development of exercise prescription in this population.

2.8 Aims

The primary aim of the studies included in this thesis was to investigate the potential for exercise as therapeutic strategy with regards to inflammation, immune function and MP characteristics in the ESRD population, with a view to further informing exercise prescription and guidelines in this patient population. The primary aim of study 1 was to characterise the MP response to 1 hour of moderate intensity aerobic exercise in order to provide information for comparison with renal populations in subsequent studies. The primary aim of study 2 was to investigate the impact of a 6-month IDC intervention on systemic inflammation and MP characteristics in a HD population, in comparison with a usual care control condition. The primary aim of study 3 was to examine the effect of a more novel exercise modality (electrical muscle stimulation) on markers of systemic inflammation in a HD population, in comparison with an intradialytic cycling condition and a usual care control condition. Finally, the primary aim of study 4 was to characterise the MP, immune and inflammatory response to 20 minutes of moderate intensity aerobic exercise acute aerobic exercise in RTRs, in comparison with a non-dialysis dependent CKD group and a healthy control group.

Chapter 3

General Methods

3.1 Research design

The results presented in this thesis derive from collaborative research between Loughborough University, the University Hospitals of Leicester (UHL), the University of Leicester and the University Hospitals of Coventry and Warwickshire (UHCW). The participants in these studies include patients recruited from the renal departments of both the UHL and UHCW NHS trusts and healthy volunteers recruited from both the local community and staff and students from Loughborough University and the University of Leicester.

The experimental chapters of this thesis are comprised of four distinct study protocols. Each study, including a summary and interpretation of the findings, is described in the relevant chapter.

Study one is an acute exercise intervention study investigating the effects of 1 hour of moderate intensity running exercise on MP phenotypes and characteristics in healthy individuals, in comparison with a resting control trial completed in a randomised crossover manner. This is described in Chapter 4.

Study two is a randomised controlled trial, using a longitudinal exercise intervention to investigate the effects of 6-months of thrice-weekly intradialytic cycling on markers of systemic inflammation and MP phenotypes in haemodialysis patients versus a no-exercise control group. This study involved patients from the UHL NHS trust and is presented in Chapter 5.

Study three is a randomised controlled trial, investigating the impact of 12 weeks of low-frequency electrical muscle stimulation of the quadriceps on markers of systemic inflammation in HD patients, in comparison with an intradialytic cycling group and a usual care control group. This study involved patients from the UHCW NHS trust and is described in Chapter 6.

Study 4 is an acute exercise intervention study investigating the effects of 20 minutes of moderate intensity walking exercise on markers of systemic inflammation, immune function and MP phenotypes in RTRs, in comparison with a non-dialysis dependent CKD population and a healthy control population. HD patients were not included in this study due to logistical purposes. This study involved both healthy participants and patients from the UHL NHS trust and is presented in Chapter 7.

Ethics

All research was given a favourable ethical opinion by the relevant governing body and conducted accordingly. The ethics committee and governing body differed depending on the population in question (i.e. patients and healthy controls) and is referred to in greater detail in each study-specific chapter.

Recruitment process

Patients

All patients were first screened for eligibility and asked if they were willing to speak to a researcher by their consultant nephrologist prior to being approached by a member of the research team. Renal transplant recipients were approached either during or after their routine appointments with their consultant nephrologist. Haemodialysis patients were approached during their regular haemodialysis treatments. The study protocol was thoroughly explained to all patients, and they were given the chance to ask any questions if necessary. Patients were given at least 48 hours to consider their participation in the research study, during which time they were given the patient information sheet and any other relevant documents to read over fully in their own time. After at least 48 hours consent was obtained by a researcher qualified in taking consent in NHS patient populations. Patients were informed of their right to withdraw at any time, and copies of their consent forms were filed in their medical notes and sent to their GPs.

Healthy participants

Healthy participants were recruited from the local community, provided they fulfilled the inclusion and exclusion criteria. They were approached via leaflets, posters and emails and had the respective study fully explained to them prior to giving informed consent.

General exclusion criteria

Exclusion criteria are summarised in table 3.1.

Exclusion Criteria for Healthy Control Participants
• **Evidence of kidney disease (e.g. urinary problems)** • **Age <18 or >80 years** • **Personal history of cardiovascular disease, metabolic disease, high blood pressure or dyslipidaemia (abnormal blood fat (triglyceride) or cholesterol)** • **Dieting or have extreme dietary habits Age <18 years** • **Diabetic** • **Current smoker** • **Clinically significant infection within the last 6 weeks** • **Taking drugs known to affect digestion, metabolism or inflammation, medical or illegal (e.g. anabolic steroids, marijuana, amphetamines, thyroid prescription drugs, corticosteroids).** • **Pregnancy** • **Physically incapable of walking for 20 minutes at a brisk pace, to be mutually decided upon by the participant and the investigators** • **Insufficient command of English to understand the patient information sheet and give informed consent**
Exclusion Criteria for Haemodialysis Patients
• **Age <18 years** • **Unable to participate in current exercise program due to perceived physical or psychological barriers** • **Unable to undergo MRI scanning (metal implants / prostheses, claustrophobia etc.)** • **Unfit to undertake exercise according to the American College of Sports Medicine guidelines** • **contraindications to exercise testing that include:** **I. Recent significant change in resting ECG that suggests significant ischaemia, recent myocardial infarction (2 weeks) or other acute cardiac event;** **II. Unstable angina;** **III. Uncontrolled cardiac dysrhythmias causing symptoms or haemodynamic compromise;** **IV. Symptomatic severe aortic stenosis;** **V. Uncontrolled symptomatic heart failure;** **VI. Acute pulmonary embolus or pulmonary infarction;** **VII. Acute myocarditis or pericarditis;** **VIII. Suspected or known dissecting aneurysm;** **IX. Acute systemic infection, accompanied by fever, body aches or swollen lymph glands.** • **Unable or unwilling to give informed consent** • **Uncontrolled blood pressure: systolic > 160, diastolic >95 during the months before enrolment** • **Excessive fluid accumulation between dialysis sessions (>3 liters), more than twice pulmonary edema over 3 months before enrolment deemed to be due to excess fluid intake** • **Haemoglobin unstable and below 9.0** • **Morbidly obese, mid-thigh circumference of more than 60cm (EMS straps limit)** **Planned kidney transplant during study period.**
Exclusion Criteria for Renal Transplant Recipients
• **Age <18 years**

- **Pregnancy**
- **Received kidney transplant less than 6 months prior to study entry**
- **Any element of study assessment protocol considered by own clinician to be contraindicated due to physical impairment, co-morbidity or any other reason**
- **Inability to give informed consent for any reason**
- **Visual or hearing impairment or insufficient command of English to give informed consent or comply with the assessment protocol**

Table 3.1. General exclusion criteria for healthy control and patient populations.

3.2 Clinical treatment

Transplant treatment

Following the receipt of a donor kidney, patients remain in the hospital until it is confirmed that the kidney is functional. In some cases this can take several days, during which they can receive supplementary dialysis if necessary. Following this the patient is discharged from inpatient care. They are then seen regularly by their consultant nephrologist – usually once a week initially but then reduced where possible to a minimum of once every 6 months. During this appointment the patient undergoes a routine blood test, can receive prescriptions for any associated side-effects or co-morbidities, and have their immunosuppressive drug regime altered where necessary. Due to the uncertain nature of the first few months following transplantation, no patient was approached until at least 6 months post-successful transplantation.

Haemodialysis treatment

Routine haemodialysis treatment continued unaffected during each research study. Prescribed medications, dietary advice and any treatment pertaining to other present medical conditions continued as normal throughout each study.

Shift patterns

Patients from all included HD units followed a similar treatment pattern and procedure. As is current general practice in the UK, unit-based HD treatment was completed for 4 hours, 3 times a week. Patients are allocated to a 'shift', either in the morning (roughly 7am-11am), afternoon (roughly noon-

4pm) or evening (roughly 5pm-9pm) that they attend thrice weekly, either on a Monday, Wednesday and Friday or on a Tuesday, Thursday and Saturday. As the first shift of every week was preceded by an extra day off dialysis treatment (the 'long break') which would consequently create extra uraemic toxin and fluid accumulation, no outcome measures were completed on these days.

Dialysis machines

The HD machines used differed between patients and dialysis units (mostly Fresenius 5008 high-volume dialysers, Fresenius, Birmingham, UK), however were all high-flux dialysers using a polysulfone membrane. The HD machines regularly check arterial and venous pressure and alarm when significant changes are detected – this ensures adequate blood flow through the vascular access throughout the entire HD shift. All patients received thrice weekly dialysis sessions each 4 hours long, and the ultrafiltration goal differed between patients and was adjusted accordingly to fluctuations in their pre-HD weight.

3.3 Outcome measures

Medical records

The medical notes of each recruited patient were accessed in order to extract their medical history, including primary diagnosis, dialysis or transplant duration and any current co-morbidities. Prescribed medications, including dosage, were recorded at the beginning and end of the study. Each HD patient had monthly blood tests taken as part of their routine clinical care to test for relevant biomarkers, which were recorded each month throughout the duration of the study where available.

Blood sampling - Renal transplant recipients and healthy controls

Blood samples from healthy participants and renal transplant recipients were taken from the antecubital vein of the forearm using a standard 21-guage butterfly needle. These samples were drawn directly into monovettes pre-treated with an anticoagulant as explained below ('Plasma Collection'), and gently inverted several times before being processed and stored for future analysis.

Blood sampling - Haemodialysis patients

All blood samples taken from HD patients were obtained during their routine dialysis treatment from their vascular access (i.e. AVF or catheter). Blood was drawn directly into pre-treated monovettes and gently inversed several times before being processed and stored for future analysis.

Physical function tests

Peak aerobic capacity (i.e. VO_2peak) was estimated using the Incremental Shuttle Walk Test (ISWT). This test requires the patient to walk up and down around two cones set 9m apart (each shuttle totals 10m once the distance of the turning circle is accounted for) in time with a progressively quickening beep from a standard CD. This is completed in 1 min stages – the first stage comprises 3 shuttles, the second stage comprises 4 shuttles and so on up to stage 12 after which the test is terminated.

Aerobic endurance was assessed using the Endurance Shuttle Walk Test (ESWT), which is completed after the ISWT. The distance completed in the ISWT dictates the speed at which the ESWT is completed. The ESWT is completed at a speed that represents 85% VO_2peak – this speed is generated by entering the ISWT distance into a conversion table. The patient walks continuously around the same set of cones at this speed in time with the beep and is encouraged to walk for as long as possible. If the patient reaches the end of the test (20 minutes) the test is terminated. This test was originally designed for patients with Chronic Obstructive Pulmonary Disease (COPD) patients (Revill et al. 1999) but has been previously used in the CKD population (Greenwood et al. 2012).

Renal function assessment

The eGFRs of the HD and transplant patients were recorded from their routine blood tests. These results were entered into medical records and were thus available for extraction by the researcher. For the healthy controls, blood samples taken on the day of testing were sent to the UHL pathology laboratory at the Leicester Royal Infirmary for testing of renal profile (eGFR, Urea, Serum Creatinine, Bicarbonate, Sodium, Potassium, Magnesium, and Phosphate). These results were sent directly to the researcher and were checked for clinically significant findings.

3.4 Laboratory techniques

Plasma collection

All monovettes were acquired from Sarstedt (Nümbrecht, Germany). Venous blood collected into K_3E monovettes (1.6 mg/ml blood) was centrifuged at 2,500 g for 10 min at 4°C. The separated plasma was removed in 500 µl aliquots and stored in Eppendorf tubes at -80°C until analysis. The remaining sample was discarded.

Venous blood, collected into monovettes containing sodium citrate as an anti-coagulant (0.106 mol/L), was first centrifuged at 2,500 g for 15 min at 20°C. The supernatant was then aliquoted into Eppendorf tubes in 1 ml measures, which then underwent a second centrifugation at 2,500 g for 15 min at 20°C. The top 90% of this sample was removed and stored in 250 µl aliquots at -80°C until analysis. The remaining 10% of the sample (containing the platelets) was discarded. This double-centrifugation procedure was successful in creating platelet-free plasma, as is displayed in Appendix 1.

Cytometric bead array (cytokines and chemokines)

Soluble cellular mediators (i.e. cytokines and chemokines) were quantitatively assessed using a Cytometric Bead Array (CBA) technique. This multiplex technique uses beads coated with antibodies that bind to the protein of interest following incubation. These beads can then be identified and allow the relative expression of each protein to be assessed by flow cytometry using beads equivalent to known standard concentrations of each protein. This allows the quantification of several analytes within a single sample. This technique can be employed either using a pre-set kit provided by the manufacturer, or by using a 'flex-set' which allows the configuration of a specific panel of analytes. Both of these techniques were employed here and are explained independently below.

Pre-set kit (chemokines)

Standards were first prepared using serial dilutions, creating dilutions ranging from the top standard (i.e. no dilution) through to a 1:256 dilution, followed by a 'zero' negative control tube (the corresponding concentrations are presented in Table 3.2). The capture beads (7.5 µm) for each analyte

were then re-conjugated into one tube and then subsequently added to each sample tube. These beads were dyed with a red dye which is excited by the red laser and produces a detectable fluorescence signal (the 'cluster parameter') - each bead population with a given fluorescence intensity represents a discrete population that will be used to measure a different analyte (Figure 3.2 B). Each bead within the same intensity population was pre-coated with a 'capture antibody' which binds with the analyte of choice – this antibody differed between each distinct bead population. Following this, 50 µL of each sample was added to the assay tubes already containing 50 µL of the mixed capture beads. 50 µL of the detection reagent (Phycoerythrin (PE)-conjugated antibody specific to the analytes of choice) was then added, and the samples were incubated in the dark at room temperature for 3 h. Addition of the detection reagent causes the creation of 'sandwich complexes' (capture bead plus analyte plus detection reagent – Figure 3.1) during this incubation period. Each discrete bead population binds to the corresponding analyte, and the fluorescent signal provided by the detection reagent is proportional to the amount and hence concentration of the bound analyte.

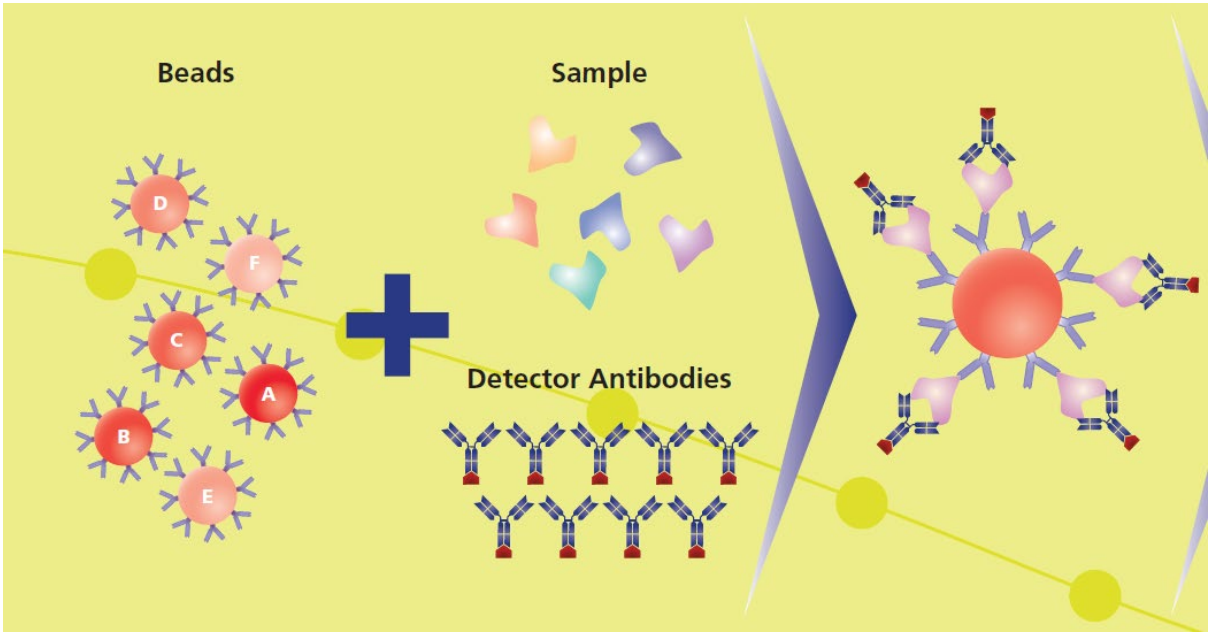

Figure 3.1. Demonstration of the sandwich complex formed during incubation, comprised of the beads, sample proteins and detector antibodies (BD Cytometric Bead Array – *Multiplexed Bead-Based Immunoassays*).

Analyte Standard Concentration	**Concentration** (pg/mL)
No standard (negative control)	0
1:256	20
1:128	40
1:64	80
1:32	156
1:16	312.5
1:8	625
1:4	1,250
1:2	2,500
Top Standard	5,000

Table 3.2. Corresponding concentrations of pre-set kit standards.

Following this 3 h incubation period during which the sandwich complexes are formed, the samples and standards were acquired on a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK) at a medium flow rate (35 µL/min) to a limit of 2100 events as instructed by the assay provider. The data was then imported for analysis to the BD FCAP Array Software Version 3.0 (BD Biosciences, Oxford, UK). Each discrete bead population corresponding to a particular analyte was first identified automatically by the software based on their distinct pre-determined fluorescence intensities as demonstrated in Figure 3.2 (the 'cluster parameter'). If a bead population was not detected automatically by the software then it was identified manually, however this was rare. The software then quantified the fluorescence intensity of phycoerythrin (PE - the 'reporter parameter'). Quantitative values of the concentrations of each analyte were obtained by inserting the mean fluorescence intensity values into the standard curve obtained from the serially diluted standards.

The main advantage of this technique over traditional ELISA methods is the reduction in time, cost and sample volume requirements. As up to 10 analytes can be measured from as little as 50µl of a single sample in one assay, the necessary sample volume is massively reduced. Similarly, completing one CBA assay is equivalent to multiple ELISAs, and typically takes less time than a single ELISA.

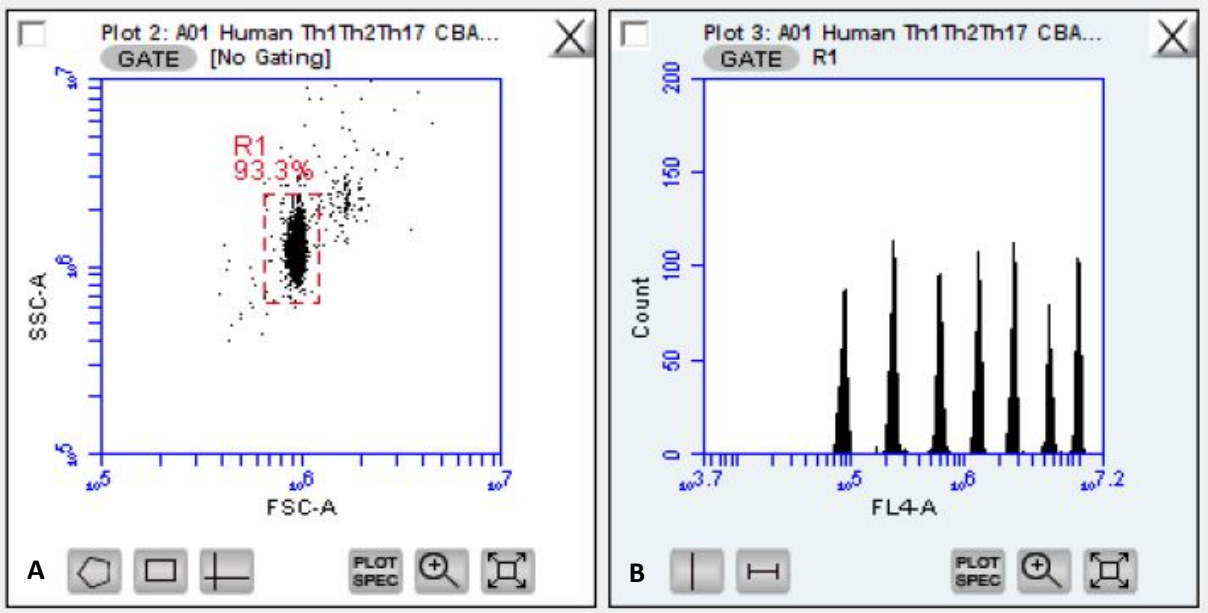

Figure 3.2. Sample data showing A) the distinct population of beads of known size (i.e. 7.5µm) and B) the discrete bead populations, distinguishable by the pre-determined fluorescence intensities measured in the FL-4 channel. This data is then exported as an FCS file and imported into the FCAP Array software for further analysis.

The minimum detectable thresholds of each cytokine/chemokine included in the pre-set kits are displayed in Table 3.3.

Pre-set Kit	Analyte	Limit of Detection (pg/mL)
Human Th1/Th2/Th17 Cytokine Kit	IL-2	2.6*
	IL-4	4.9*
	IL-6	2.4*
	IL-10	4.5*
	TNF-α	3.8*
	IFN-γ	3.7*
	IL-17A	18.9*
Human Chemokine Kit	CXCL8/IL-8	0.2
	CCL5/RANTES	1.0
	CXCL9/MIG	2.5
	CCL2/MCP-1	2.7
	CXCL10/IP-10	2.8

Table 3.3. The minimum detectable thresholds of the cytokines/chemokines in the panels analysed in the pre-set kits. * indicates the minimum detection limits that are too high for the samples used in this thesis – as such these analytes were assessed with the flex sets as described below.

Flex-sets (cytokines)

For the analytes that fell under the minimum detectable thresholds in the pre-set kits (Table 2.3), a high sensitivity flex set was configured and implemented. This multiplex follows the same principle as that of the pre-set kits explained above, however requires the compilation of the panel of desired analytes by the researcher.

Standards were first prepared using serial dilutions, creating dilutions ranging from the top standard (i.e. no dilution) through to a 1:729 dilution (1/3 serial dilutions), followed by a 'zero' negative control tube (the corresponding concentrations are presented in Table 3.4). The capture beads (7.5 µm) for each analyte were then re-conjugated into one tube and then subsequently added to each sample tube. Differing to the pre-set kits, these beads were coated with a dye that, once excited and fluorescing, is detected by two separate detectors (i.e. two 'cluster parameters') thus creating a three-dimensional matrix from which to identify each discrete bead population, corresponding to each analyte in question (Figure 3.3). To allow for this on the Accuri C6 (which is typically set up so that 3 detectors detect signals excited by the blue laser and 1 detector detects signals excited by the red laser) a selectable lasers module was installed to reassign 1 extra detector from the blue to the red laser (in combination with a change in optical filter) to therefore allow the red dye to produce a signal which provides 2 cluster parameters.

Analyte Standard Concentration	**Concentration** (fg/mL)
No standard (negative control)	0
1:729	274
1:243	823
1:81	2,469
1:27	7,407
1:9	22,222
1:3	66,667
Top Standard	200,000

Table 3.4. Corresponding concentrations of flex-set standards.

This technique therefore required several incubations. Firstly, the capture beads were incubated with the unknown sample for 2 h. Secondly, the detection reagent (PE – the 'reporter parameter' which excites a specific detector and corresponds to the amount of bound analyte) was added for a further 2 h incubation. Lastly, as a high sensitivity assay was necessary for all cytokines, an enhanced sensitivity detection reagent was added for a final 1 h incubation. The samples were then acquired and analysed in an identical manner to those included in the pre-set kits, as explained earlier.

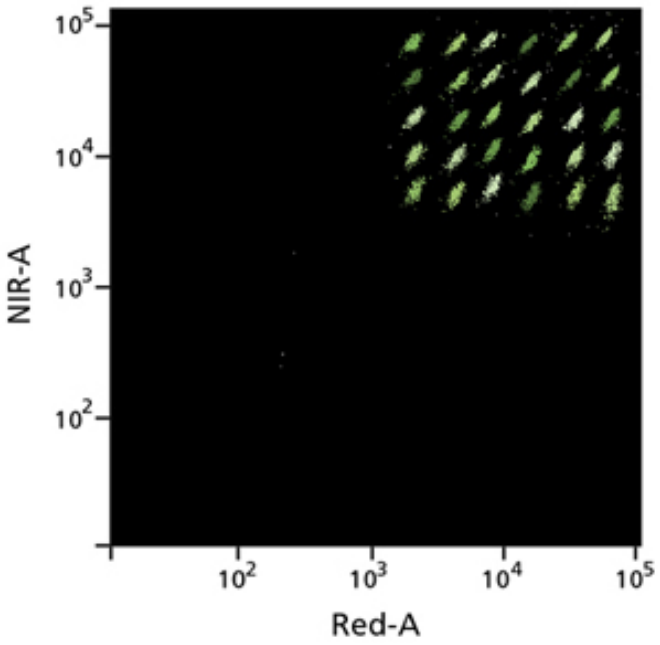

Figure 3.3. Demonstrating the 3-dimensional cluster parameter matrix produced by the flex sets, creating discrete bead populations (30 populations used here – the maximum number possible using the flex set), each corresponding to a specific analyte which can be further analysed by the FCAP software to determine its degree of binding with PE and determine the concentration of that analyte.

This technique provides a theoretical minimum detectable threshold of 0.274 pg/mL for all analytes. Where results still fell below the minimum detectable threshold of the flex-set analysis technique, the samples were omitted and not included in the subsequent statistical analyses. Patient samples were included in analysis if samples from all time-points returned valid and detectable results. The number of samples included in the analyses are presented with each presented variable within each study. This

technique typically produces reported inter- and intra-assay coefficient of variations of below 6% for typically measured analytes, such as IL-2, IL-6, IL-10, TNF-α and IFN-γ (Morgan et al. 2004). However, analysis of duplicate samples completed on a cohort of the samples included in this thesis (n = 61, after removal of invalid samples) produced a mean intra-assay coefficient of variation of 10.08% for this technique (raw data included in appendix 2). Duplicate analyses were only completed on this cohort for the determination of intra-assay variation and were not completed on all samples included in this thesis. This may represent a limitation of the results in this thesis gained by the cytometric bead array technique, as any effect (e.g. in response to an exercise intervention) that is not greater than the observed variation inherent in the technique may not be detected, increasing the chance of committing a Type II error. Whilst it is standard practice to complete the analysis of soluble proteins in duplicate (or even triplicate) in order to account for intra-assay variation and provide a more precise result, the manufacturers of the employed cytometric bead array technique (BD Biosciences) do not recommend completing analyses in duplicate. This is because, as every bead effectively represents a well on a traditional ELISA (i.e. a pool of a specific antibody ready to bind with the protein of interest, but coated onto a bead instead of residing in a well) and every sample is acquired until roughly 300 beads per analyte have been recorded and quantified, duplicate analysis is not necessary as the final calculated concentration is the average of these 300 beads.

Microparticle analysis

The full protocol used for flow cytometric analysis of circulating MPs and their pro-thrombotic potential can be found in Appendix 3. Before analysis of MPs began, an antibody volume optimisation experiment was completed to ensure the optimal volume of antibody was used with regards to minimising volume used whilst maximising staining and ensuring consistency across each cellular derivation (Appendix 4). Similarly, 'Megamix' beads (BioCytex, Theale, UK) of known size (0.5 µm, 0.9 µm and 3.0 µm) were analysed on the BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK) to determine the thresholds for MP size gating (Figure 3.4). The population of 'All MPs' was assessed using size gating and Annexin-V expression (An-V^+) to determine the number of MPs which were AnV^+. Microparticle derivations were categorised as platelet-derived ($CD42b^+$), neutrophil-derived ($CD66b^+$), monocyte-derived ($CD14^+$) and

endothelial cell-derived ($CD144^+$). The pro-thrombotic potential of each MP derivation was assessed using TF ($CD142^+$) expression. Corresponding isotype controls were used to account for non-specific staining. Platelet-free plasma samples, originally stored at -80ºC in 250 µl aliquots (as explained above) were thawed at room temperature, and then centrifuged at 18,000 g for 30 min at room temperature. Following this, 90% of the supernatant (i.e. 225 µl) was removed and discarded – this volume was then replaced with an identical volume of MP buffer (the exact composition of which can be found in the protocol in Appendix 3). This was then centrifuged again at 18,000 g for 30 min at room temperature. Finally, 90% of the supernatant was removed and discarded again, and replaced by an equal volume of MP buffer, after which a pipette was used to gently redistribute the MPs in the buffer using 4 depressions. 10 µl of this sample was then incubated in the dark at room temperature for 25 mins with the appropriate antibodies (Appendix 3). The isotype controls used were anti-IgG1k PE, anti-IgG1k APC, anti-IgMk PE and anti-IgG2ak PE. The antibodies used for assessing MPs were anti-AnV FITC, anti-CD42b PE, anti-CD66b PE, anti-CD14 PE, anti-CD144 PE and anti-CD142 APC. All antibodies were purchased from BD Biosciences (Oxford, UK) apart from CD142-APC, which was purchased from Biolegend (London, UK).

Following incubation, MP samples were redistributed in an inert buffer (the exact composition of which can be found in the protocol in Appendix 3), and then analysed on a C6 flow cytometer. All samples were collected for 2 mins at a 'slow' flow rate (14 µl/min) and collected in scatter and fluorescence plots. These plots had been set up previously using unstained samples to position and size the gates, and adjustment of the laser voltage sensitivity to elicit appropriate colour compensation. This allowed automatic analysis of data and removed the need for subjective post-acquisition gating to enumerate MPs. The gating strategy used is displayed in Figure 3.4 and 3.5. Therefore, no post-acquisition analysis was necessary – the counts and concentrations of each MP phenotype were recorded directly from the acquisition software.

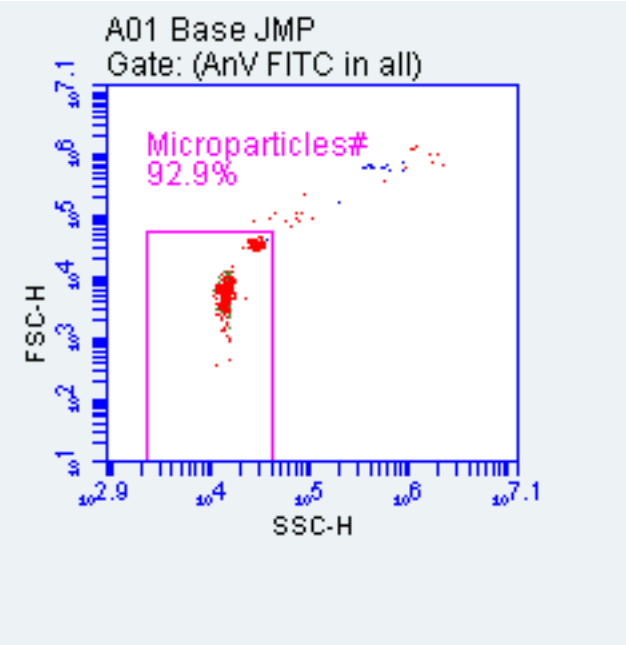

Figure 3.4. Data obtained by flow cytometric acquisition of megamix beads. Two distinct populations (0.5 and 0.9 µm beads, shown in red) are visible, and thus the gate can be set to include these populations whilst excluding the 3.0 µm population (not visible here). Based upon the position of these bead populations, the maximum and minimum size thresholds for MP analysis can be set across all subsequent MP analyses. Adjusting the minimum size threshold to include these populations creates a quantifiable 'trigger parameter' (i.e. a particle will only trigger an event if it falls above the threshold) – this minimum threshold, following appropriate adjustment, can be applied to all future MP size gates (see Figure 2.5a). Based on the manufacturer's recommendations (BioCytex, UK), these thresholds were set to acquire samples between 0.3 and 1.0 µm. Despite MPs ranging in size from 0.1 to 1.0 µm, a minimum threshold of 0.3 µm provides an optimal compromise between MP analysis and background exclusion (i.e. it sufficiently removes the noise floor inherent in the system whilst minimising the removal of the particles of interest). This bead-based size gating standardisation procedure has been implemented successfully in previous research in the optimisation of MP analysis (Robert et al. 2009).

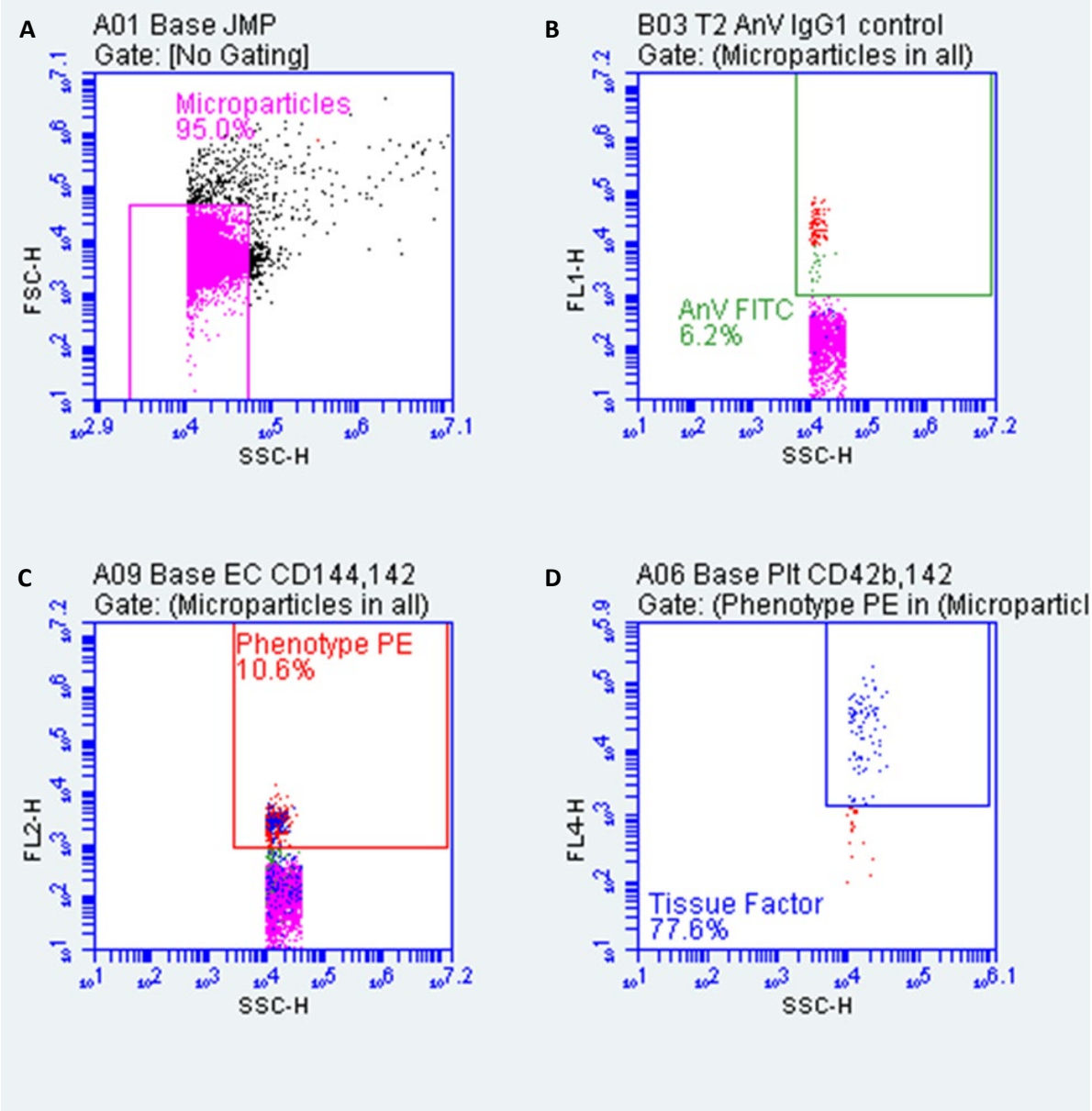

Figure 3.5. Demonstration of the gating strategy used to characterise MPs. A: 'All MPs' based on size, set using mega-mix beads of known size. The minimum trigger threshold of 0.3 µm is necessary to exclude the noise floor inherent in all cytometers, hence why the visible MP population (known to be comprised of particles ranging from 0.1 to 1.0 µm in diameter) is cut off at around 10^4 on the X axis. B: 'All MPs' based on Annexin-v expression. C: Phenotype marker expression, used to quantify MPs of different cellular sources. D: Pro-thrombotic potential, assessed by Tissue Factor expression. The gates on graphs B, C and D have been appropriately sized and positioned using unstained samples to distinguish negative vs positive staining – therefore all events within the gates are considered to be positively stained and therefore the particle of interest.

This sample preparation and analysis procedure was based on recommendations in previous recent literature which highlight the requirements and optimal conditions necessary for MP analysis (Dey-Hazra et al. 2010; van der Hyde et al. 2011; Szatanek et al. 2017). Analysis of duplicate samples completed on a cohort of the samples included in this thesis (n = 72) produced a mean intra-assay

coefficient of variation of 13.88% for this technique (Appendix 2).Similar to the results gained via cytometric bead array, duplicate analysis was only completed on a cohort of samples in order to obtain the coefficient of variation results, and was not completed on all the samples presented in this thesis. This may represent a limitation given the large reported coefficient of variation (as described earlier). However, due to the significant time and cost-intensive nature of the MP analysis procedure, duplicate analysis on all included samples was not considered to be feasible.

3.5 Statistical analysis

The study-specific statistical analysis methodology is presented in each chapter. Normality was assessed using the Shapiro-Wilk test. Non-normally distributed data was logarithmically transformed, after which the effectiveness of the transformation was assessed prior to completing parametric testing. All data was either normally distributed or successfully transformed and as such no non-parametric testing was completed. Data that was analysed in a transformed format is noted where that data is presented (i.e. in a table or figure). Where appropriate, one-way analysis of variance (ANOVA) or independent samples t-tests were completed to determine baseline differences between groups. Repeated or mixed-design ANOVAs were completed where necessary. If Mauchly's test of sphericity was violated when completing ANOVA, the degrees of freedom were corrected using the Greenhouse-Geisser method and the appropriate p value reported. If appropriate *post-hoc* testing was completed and adjusted for multiple comparisons using the Bonferroni correction to elucidate the nature of the significance whilst limiting alpha inflation and thus the risk of Type I error (McHugh 2011).

All statistical analyses were performed using the Statistical Package for the Social Sciences (IBM SPSS v. 23, IBM, New York, USA). All graphs were created using GraphPad Prism (v.6 GraphPad Software Inc., CA, USA). All population or patient descriptive data is presented as 'mean ± standard deviation' (SD), whilst outcome data is presented as 'mean ± standard error of the mean' (SEM) when presented in figures (for clarity) and 'mean ± SD' when presented in tables or the main body of text. Significance was accepted at the $p < 0.05$ level. Effect sizes were calculated using partial eta squared (η^2) and were considered as a small (0.2), medium (0.5) or large (0.8) effect (Cohen 1988). Sample size calculations

were completed using GPower version 3.1 (Düsseldorf, Germany) (Faul et al. 2007). Where appropriate, in response to non-significant findings, post-hoc sample size calculations were completed to calculate the required sample size necessary to detect significant findings (provide an alpha of .05 and statistical power of .95) given the presented effect size (η^2).

Chapter 4

Study 1

The acute effects of moderate intensity exercise on microparticle characteristics in healthy individuals

4.1 Abstract

Microparticles (MPs) are extracellular vesicles shed upon cellular activation or apoptosis that possess pro-thrombotic functions via Tissue Factor (TF) expression. Aerobic exercise (AE) may impact circulating MP kinetics and concentrations, though recent research has found conflicting results. This study investigated the impact of AE or rest on MP phenotypes and pro-thrombotic potential in a population of healthy individuals.

Fifteen healthy males (age 22.9 ± 3.3 years; body mass 81.9 ± 11.4 kg; VO_2 max 54.9 ± 6.5 mL·kg·min^{-1}; mean ± SD) completed 1 hour of AE (70% VO_2max) at 9am, and consumed a standardised meal (1170 kcal, 43% CHO, 17% PRO, 40% fat) at 10:45am. Venous blood samples were taken at 9am, 10am and 11:30am. Control trials were identical but included no exercise. MP phenotypes (platelet-derived – CD4b$^+$, neutrophil-derived – CD66b$^+$, monocyte-derived – CD14$^+$, and endothelial cell-derived – CD144$^+$) and TF (CD142$^+$) expression were assessed by flow cytometry, whilst MP presence was confirmed using nanoparticle tracking analysis (NTA). Statistical analysis was completed using IBM SPSS version 23.

Outcome data are presented as mean ± SD**.** Effect sizes are presented as η^2 (0.2 = small, 0.5 = moderate, 0.8 = large, Cohen, 1988). NTA revealed no significant changes in MP concentration or diameter in response to time or trial. Total MP number (assessed via flow cytometry) and all other phenotype counts remained unaffected by the exercise bout. The % of TF+ platelet-derived MPs reduced from 9am to 10am (44.0 ± 21.3 to 21.5 ± 9.4%, p = .001, η^2 = .582) in the exercise trial, but remained unchanged in the control trial (36.8 ± 18.2 to 34.9 ± 15.1%, p = .972). The % of TF+ neutrophil-derived MPs reduced from 9am to 11:30am (42.3 ± 17.0 to 25.1 ± 14.4%, p = .048, η^2 = .801) in the exercise trial, but remained unchanged in the control trial (28.5 ± 15.9 to 32.2 ± 9.7%, p = .508).

Moderate intensity AE seems to have little effect on absolute circulating MP phenotype counts in this healthy population. However, AE induced a reduction in the % of platelet and neutrophil MPs that express TF, suggesting a mechanism through which AE can reduce cardiovascular risk via reduced TF-stimulated coagulation and thrombosis. This effect requires more investigation in clinical populations, particularly those at greater risk of cardiovascular disease. The assessment of the pro-thrombotic

potential of MPs in response to AE training in clinical populations could provide another method to assess intervention efficacy, and ultimately be used to tailor individual exercise programmes in order to greater reduce cardiovascular disease risk.

4.2 Introduction

Microparticles (MPs) are small (0.1-1.0 μm) extracellular vesicles shed upon cellular activation or apoptosis and can act as biomarkers for inflammation and immune cell and endothelial dysfunction. MPs can also influence inflammation, oxidative stress and coagulation and thrombosis through several pathways (e.g. by promoting immune cell cytokine secretion, reducing endothelial NO release, or increasing TF bioavailability to drive thrombus formation) (Ando et al. 2002; Piccin, Murphy, and Smith 2007; Polgar et al. 2005). MPs are shed by a variety of cell types and express similar surface antigens to those of their parent cell. As such, MP phenotypes can be quantified using laboratory techniques, providing evidence on the health of the cell in question and providing potentially useful prognostic information with regards to pathophysiological processes.

Recent research has investigated the effects of both acute aerobic exercise and chronic aerobic exercise training on MP kinetics in healthy participants. However, the findings remain conflicting; some studies report increased post-exercise MP numbers of platelet origin, particularly after strenuous exercise (progressive maximal cycle ergometer test) (Chaar et al. 2011; Chen et al. 2015, 2010; Chen et al. 2013; Sossdorf et al. 2010), which suggests a pro-thrombotic effect due to the high TF expression usually found on platelet-derived MPs (Del Conde et al. 2005; Diamant et al. 2002). Mechanical activation of platelets (due to increased haemodynamic stress) and thus accelerated MP shedding is proposed to be the cause of this. Conversely, other studies have found no change in endothelial cell or platelet-derived MP numbers following high-intensity (100% peak power output) cycling (Guiraud et al. 2013) or even shown a reduction in circulating endothelial cell-derived MP numbers following cycling of various intensities (55-100% peak power output) (Wahl et al. 2014). Further investigation is required to fully elucidate these effects.

Therefore, the aim of this study was to characterise the effect of prolonged (1 hour) moderate intensity aerobic exercise on phenotype-specific MP concentrations and TF expression in healthy participants. This will provide useful information for the basis of further research, for instance in clinical populations

who would benefit from aerobic exercise participation. It is hypothesised that the exercise will increase circulating MP numbers but reduce their TF expression.

4.3 Methods

Ethics

A favourable ethical opinion was obtained from the Loughborough University Human Participants Ethics Sub-Committee prior to study initiation (R15-P128). Participants were informed both in writing and verbally of the study protocol and any risks associated with their participation.

Recruitment

Participants were recruited from the University and local community via email or poster and leaflet distribution. Participants were informed of their right to withdraw from the study at any time and were given the opportunity to ask any questions they might have about their study participation. Following this, participants provided written informed consent in the presence of a trained researcher.

Inclusion Criteria:

1. Male
2. Aged 18 or older
3. Able to run continuously for 60 minutes at a moderate intensity (70% of VO_2 max)

Exclusion Criteria:

1. Unstable weight (>3kg change in body mass over the last 3 months)
2. Current smoker
3. Current cardio-metabolic disease or co-morbidity
4. Any food allergies
5. Currently dieting
6. Currently regularly taking any prescribed medications

Preliminary assessments

Before the completion of the main trials, each participant attended the laboratory in order to complete preliminary assessments. These assessments included questionnaires to assess health status, dietary habits, food preferences, habitual physical activity and measures of anthropometry. The questionnaires were completed to further confirm eligibility and to ensure the provided meals (as explained below) were suitable for each participant. Body composition was analysed using skinfold measurements – the sum of seven skinfolds was used to determine percentage body fat (Siri 1993).

During the assessment session, two exercise tests were completed. Each participant first completed a submaximal incremental treadmill running test (4 stages of 4 minutes each at a comfortable pace but increasing gradient) followed by maximum oxygen uptake treadmill running test (comfortable running pace, gradient increased by 1%/min until volitional exhaustion), separated by 20-30 minutes of rest. During each test, frequent expired gas samples were collected into Douglas bags (Plysu Protection Systems, Milton Keynes, UK) to determine oxygen consumption and carbon dioxide production. These measures were used to determine the running speed required to elicit 70% of the maximal oxygen uptake for each participant, which was subsequently used in the exercise visit in the main trials as the required running pace.

Main Trials

Participants were given at least 5 days to recover following the preliminary exercise tests before completing the main trials. Participants completed one exercise and one control trial, separated by at least 5 days, in a randomised crossover design (www.randomization.com).

Participants were asked to standardise dietary intake 24 hours prior to each main trial – this was accomplished via weighed food diaries and the provision of a standardised meal to be consumed within 7-8 pm the evening before (see below). Caffeine and alcohol consumption and strenuous exercise were not permitted during these 24 hours.

For each main trial, participants arrived at the laboratory in the morning (8 am) in a fasted state (only water for the previous 12 hours). A cannula was inserted into the antecubital vein upon arrival, 1 hour prior to the first venous blood sample collection in order to allow acclimatisation to the study environment and cannula (Chandarana et al. 2009).

For the exercise trial, following the first venous blood sample, participants ran continuously for 60 minutes on a treadmill set to a speed predicted to elicit 70% of VO_2 max. Expired air samples were collected for one minute every 15 minutes to measure oxygen consumption – running speed was adjusted accordingly if oxygen consumption differed from 70% of maximum. Heart rate and rating of perceived exertion were recorded every 15 minutes. A second blood sample was taken immediately post-exercise.

After exercise, participants rested for the remainder of the trial. A standardised meal was provided at 10:45 am (see below) and a final venous blood sample was taken at 11:30 am (Schematic of exercise, meal and blood samples in Figure 4.1). In the control trial, identical procedures were followed except the participant remained rested for the entire trial.

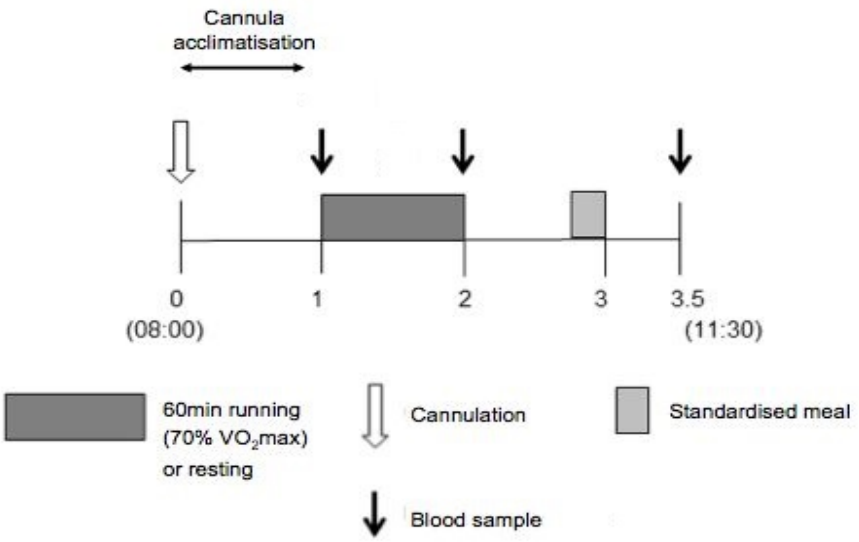

Figure 4.1. Trial schematic demonstrating the timings of cannulation, blood sampling, exercise and meal consumption.

Standardised Meal

The provided meal to be consumed the evening before each main trial consisted of a pepperoni pizza (1169 kcal, 48% CHO, 18% PRO, 34% FAT). The standardised breakfast consumed during the main trials consisted of a ham and cheese sandwich, chocolate milkshake and a chocolate bar (1170 kcal, 43% CHO, 17% PRO, 40% FAT). Participants were asked to consume this meal within 15 minutes.

Microparticle Analysis

For an in-depth description of the flow cytometry methodology employed to analyse MPs, see Chapter 3. Briefly, venous blood samples collected into sodium citrate-treated (0.106 mol/L) were double-centrifuged (15 min, 18,000g, 20°C – supernatant aliquoted and centrifugation repeated) to create platelet-free plasma and stored at -80°C for later batch analysis. Plasma was thawed at room temperature, and MP phenotypes were analysed via flow cytometry (BD Accuri C6, BD Biosciences, Oxford, UK). MPs were characterised based on size, Annexin-V expression (FITC anti-AnV) and their expression of CD42b (PE anti-CD42b - platelet-derived MPs), CD66b (PE anti-CD66b - neutrophil-derived MPs), CD14 (PE anti-CD14 - monocyte-derived MPs) or CD144 (PE anti-CD144 - endothelial cell-derived MPs). MP pro-thrombotic potential was assessed by TF (APC anti-CD142) expression by each phenotype.

Given the recent recommendations that suggest two techniques be used to detect and analyse circulating MPs, NTA was also completed on a cohort (n = 5) of the participants as the combination of NTA and flow cytometry is the current gold-standard methodology for MP analysis (Szatanek et al. 2017) (NTA was not completed on all 15 participants due to logistical constraints). Platelet-free plasma samples were suspended in phosphate-buffered saline and visualised using a Nanosight nanoparticle analyser (Malvern, UK). This apparatus uses a laser arrangement to elicit particle scatter in suspended MPs, which can be visualised using a microscope and high-sensitivity camera to create an image of individual MPs within a sample (Figure 4.2). The sample is injected using a syringe and an automatic pump controls the speed of sample entry into the visualisation prism. As the sample moves across the prism, the camera films the particles over the course of 30 seconds – this is repeated five times. The particles randomly oscillate within the sample as they move across the prism, and the degree of

oscillation is proportional to the particle size (more oscillation = smaller particle) due to the principle of Brownian Motion (Uhlenbeck and Ornstein 1930). As temperature can influence this relationship, ambient temperature was kept constant within the laboratory (20-21 °C) – this was also monitored by the Nanosight system. During analysis, the software identifies the particles and then tracks their motion over the course of 750 frames for each of the five 30 second videos. As this results in analysis of 3750 frames, any abnormally large or aggregated particles have minimal impact on the end results. The analysis of each video provides values for MP concentration and mean MP diameter – this is then averaged across the five videos to provide final results for each sample. Following sample analysis the prism is flushed with phosphate-buffered saline to remove the particles, after which the next sample is injected into the prism.

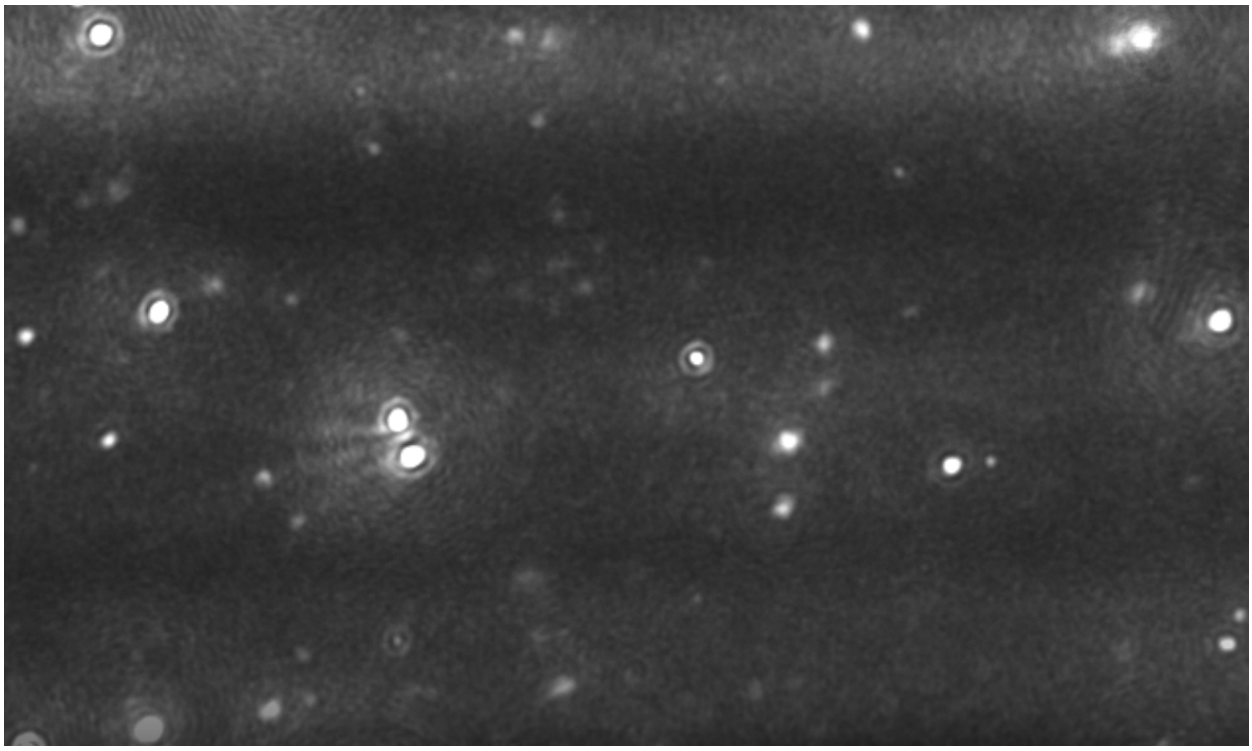

Figure 4.2. MP visualisation using the Nanosight system. Each particle is excited by a laser and visualised with a high-sensitivity camera, then tracked during post-video acquisition analysis.

Running duplicate analyses on the samples included in this chapter produced mean coefficients of variation of 5.21% and 3.55% for the MP concentration and diameter results, respectively.

This technique is able to visualise particles below the minimum threshold provided by flow cytometry (0.3 µm), however cannot provide the same qualitative information (i.e. cellular derivation or protein

expression). Similarly, the flow cytometry technique can give qualitative information on particles greater than 0.3 µm but cannot be used to comment on the concentration or characteristics of particles below 0.3 µm. As such, the NTA technique will be used to report on the whole reported size range of MPs and the flow cytometry technique used to report on MPs over 0.3 µm in diameter (labelled 'MPs > 0.3').

Statistical Analysis

Any non-normally distributed data (i.e. Shapiro-Wilks of $p < .05$) was logarithmically transformed to allow the use of parametric testing. All data was either normally distributed or successfully transformed – data which was transformed for analysis is indicated when it is presented. The impact of the exercise bout was assessed using a repeated measures ANOVA. Partial eta squared (η^2) was used to estimate effect sizes for all ANOVAs. Effect sizes were classified as small (0.2), medium (0.5) and large (0.8) (Cohen 1988). If a trial*time effect was detected, a paired samples t-test was completed between groups at each time-point to elucidate the effect. For all analyses, $p < .05$ was considered statistically significant. All descriptive data are presented as 'mean ± SD' whilst outcome data is reported as 'mean ± SEM' when presented in a figure, and 'mean ± SD' when presented in a table or discussed in the text. All statistical analysis was completed on IBM SPSS version 23.0 (Chicago, Illinois).

Acknowledgement of secondary analysis

This study was completed at Loughborough University by Fernanda Reistenbach-Goltz under the supervision of Dr David Stensel and his research team. As such, this team was responsible for study design, gaining ethical approval, participant recruitment and completion of outcome assessments. I was responsible for all laboratory analysis of MPs on stored samples, including all data extraction, processing and subsequent statistical analysis.

4.4 Results

Participants

Fifteen healthy male volunteers participated in the study. Their anthropometric, body composition and exercise performance results are presented in Table 4.1.

	Mean	Standard Deviation
Age (years)	22.9	3.3
Height (cm)	181.7	6.1
Body Mass (kg)	81.9	11.4
BMI ($kg \cdot m^2$)	24.8	3.0
Waist Circumference (cm)	84.3	6.9
Body Fat (%)	13.1	5.9
VO_2 max ($mL \cdot kg \cdot min^{-1}$)	54.9	6.5

Table 4.1. Participant anthropometric, body composition and exercise performance results.

Nanoparticle tracking analysis

The data obtained using NTA displayed no time*trial interaction effect for MP concentration ($p = .392$, $\eta^2 = .111$). This is displayed in Figure 4.3.

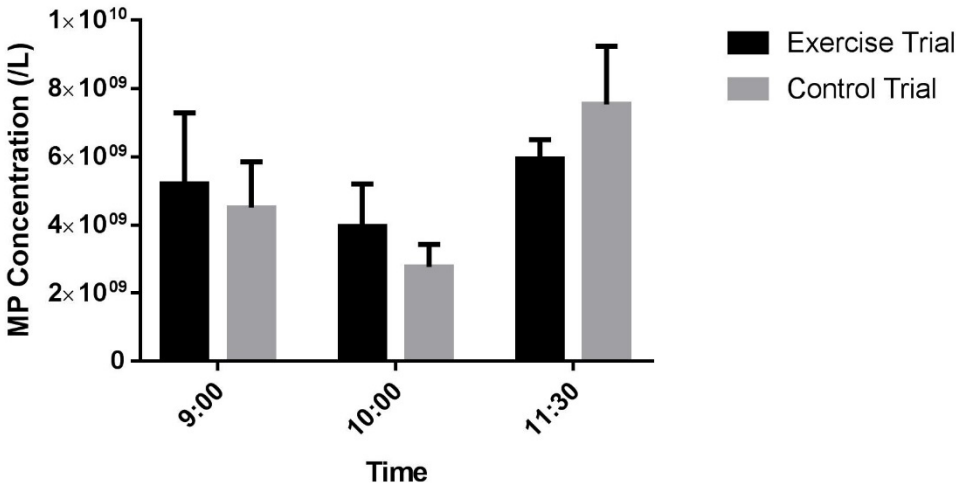

Figure 4.3. MP concentration obtained using NTA (n = 5). Data are presented as 'mean ± SEM'. This data was normally distributed.

Similarly, no time*trial interaction effect (p = .206, η^2 = .179) was exhibited for mean MP diameter. This is displayed in Figure 4.4. Post-hoc power calculations suggested that a sample size of n = 24 would be necessary to elicit a significant interaction effect.

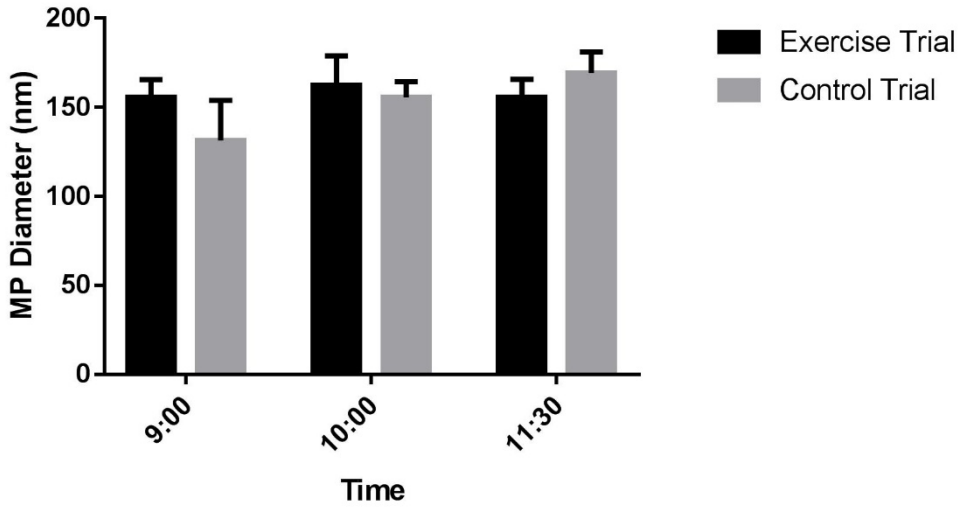

Figure 4.4. Mean MP diameter obtained using NTA (n = 5). Data are presented as 'mean ± SEM'. This data was normally distributed.

Microparticle phenotypes – flow cytometry (MPs > 0.3 µm)

'Total MPs' (i.e. quantified by size only) showed no time*trial interaction effect (p = .127, η^2 = .071) (Figure 4.5). The number of Annexin-V positive MPs (as a marker of phosphatidylserine expression) was unaffected by time (p = .567, η^2 = .013) and no time*trial interaction effect was exhibited (p = .065, η^2 = .113). Post-hoc sample size analysis suggested sample size of n = 36 would be necessary to elicit a significant interaction effect in total MP number.

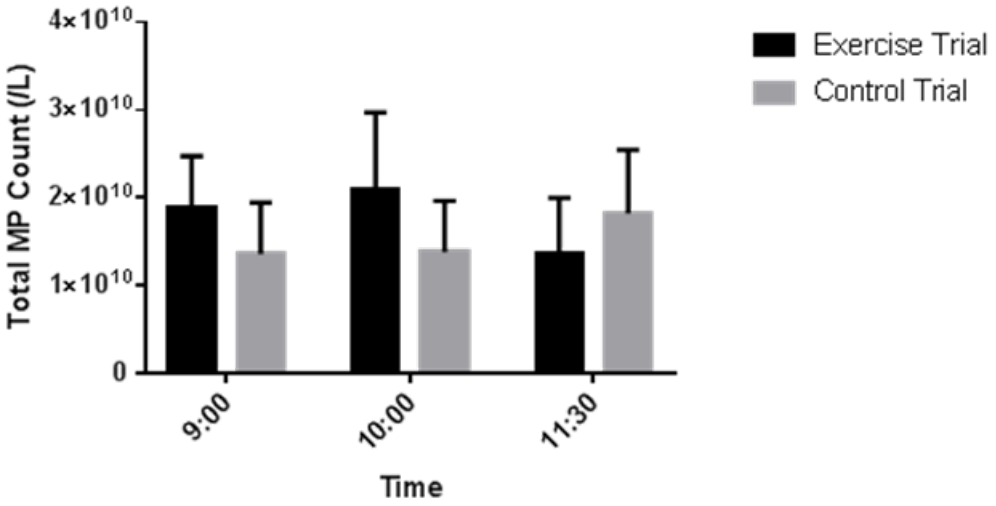

Figure 4.5. Total MP number at all 3 time-points in both exercise and control trials (MPs > 0.3 µm). (n = 15). Data are presented as 'mean ± SEM'. This data was logarithmically transformed prior to analysis.

The number of all phenotype-specific MPs displayed no significant time*trial effect ($p \geq .160$, $\eta^2 \leq .063$). Post-hoc analysis suggested a sample size of n = 40 would be necessary to detect significant changes in phenotype-specific MP numbers. Similarly, the number of each MP phenotype as a percentage of total MP count was exhibited no significant time*trial interaction effect ($p \geq .395$, $\eta^2 \leq .027$).

Microparticle procoagulant potential

The number of TF^+ platelet, neutrophil and endothelial cell-derived MPs displayed no significant time*trial interaction effects ($p \geq .069$, $\eta^2 \leq .094$) (n = 28 necessary to detect changes).

However, the number of TF^+ platelet-derived MPs as a percentage of the total platelet-derived MP count did display a significant interaction effect ($p = .001$, $\eta^2 = .216$). Further analysis revealed a reduction from 9am to 10am (44.0 ± 5.5 to 21.5 ± 2.4%, $p = .001$, $\eta^2 = .582$) in the exercise trial, but no change in the control trial (36.8 ± 4.7 to 34.9 ± 3.1%, $p = .972$, $\eta^2 = .076$). This is displayed in Figure 4.6a. Similarly, the number of TF^+ neutrophil-derived MPs as a percentage of the total neutrophil-derived MP count displayed a significant interaction effect ($p = .002$, $\eta^2 = .465$), characterised by a reduction from 9am to 11:30am (42.3 ± 4.4 to 25.1 ± 3.8%, $p = .048$, $\eta^2 = .801$) in the exercise trial, but no change in the

control trial (28.5 ± 4.1 to 32.2 ± 2.5%, p = .508, η^2 = .076). This is displayed in Figure 4.6b. However, the number of TF^+ monocyte-derived MPs and endothelial cell-derived MPs as a percentage of their total respective phenotype count exhibited no time*trial interaction effects (p ≥ .113, η^2 ≤ .103). This is displayed in Figure 4.7a and 4.7b.

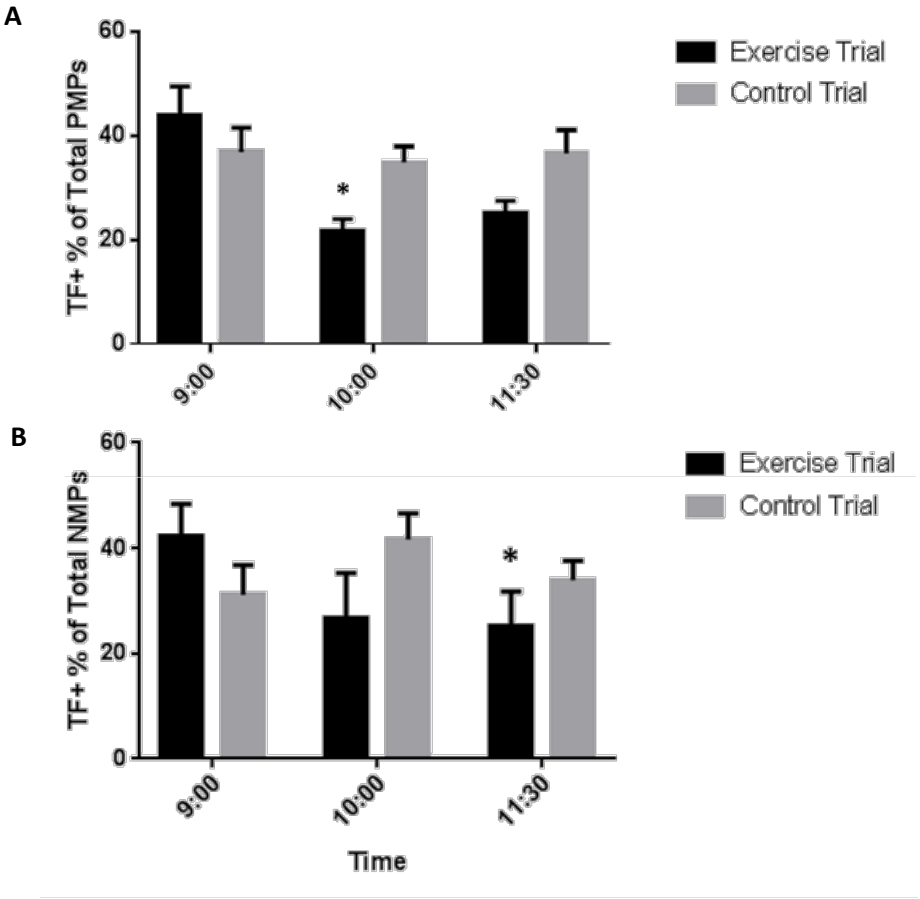

Figure 4.6. TF^+ platelet-derived MP (PMP) number as a % of total PMP number (A) and TF^+ neutrophil-derived MP (NMP) number as a % of total NMP number (B) (MPs > 0.3 µm) (n = 15). Data are presented as 'mean ± SEM'. * = significantly different from baseline (9:00) (A: p = .001, B: p = .048). This data was normally distributed and thus not transformed prior to analysis.

The data for the remaining MP results not presented graphically above (MPs > 0.3 µm), including phenotype and TF expression data, is presented in Table 4.2.

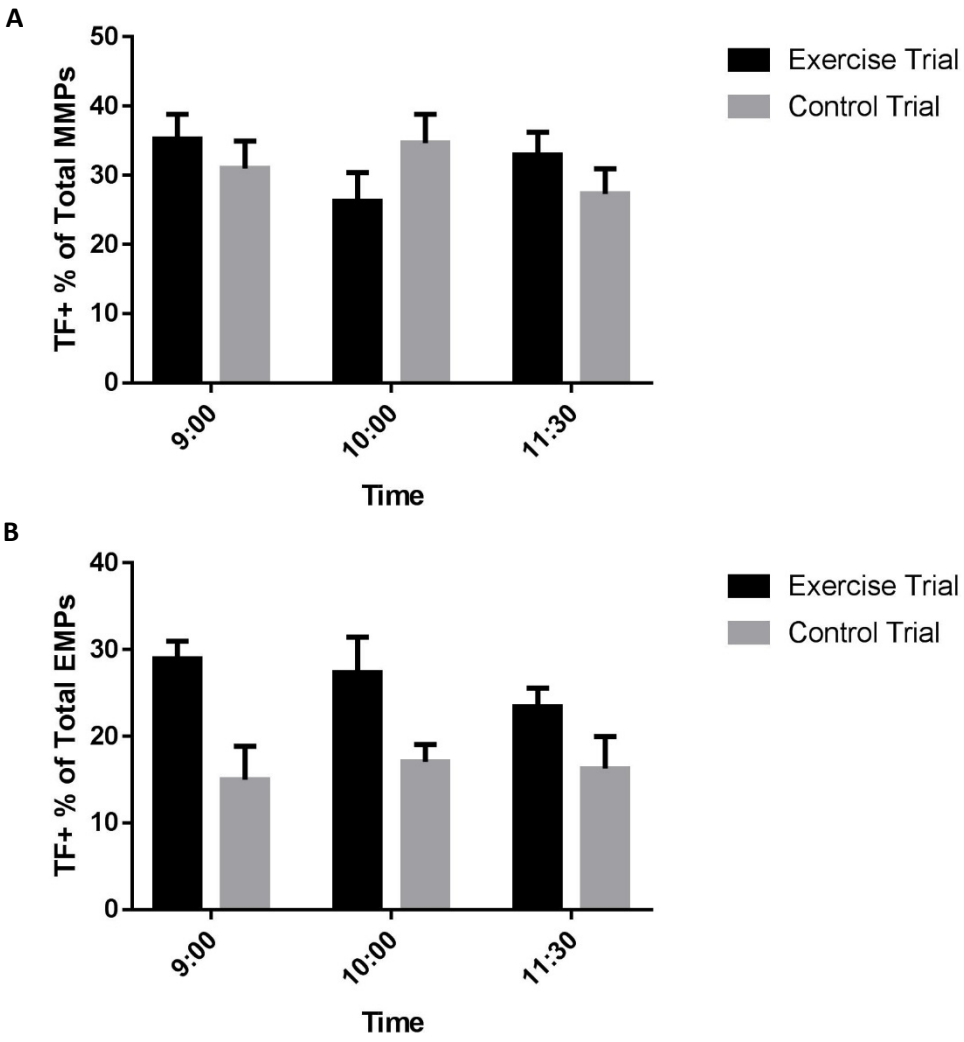

Figure 4.7. TF^+ monocyte-derived MP (MMP) number as a % of total MMP number (A) and TF^+ endothelial cell-derived MP (EMP) number as a % of total EMP number (B) (MPs > 0.3 µm) (n = 15). Data are presented as 'mean ± SEM'. This data was normally distributed and thus not transformed prior to analysis.

MP Variable	Exercise Trial			Control Trial		
	9:00	10:00	11:30	9:00	10:00	11:30
Annexin-V$^+$ Number (10^7/L)	6.16 ± 4.89	13.61 ± 5.52	7.79 ± 3.46	13.66 ± 27.10	4.50 ± 3.78	6.76 ± 3.46
CD42b$^+$ PMP Number (10^7/L)	7.78 ± 5.48	6.08 ± 3.38	5.97 ± 2.37	7.15 ± 5.25	6.86 ± 3.82	15.98 ± 2.71
CD42b$^+$ PMP % of Total (%)	1.72 ± 2.37	2.79 ± 2.85	2.07 ± 1.85	2.48 ± 3.13	3.11 ± 2.94	6.84 ± 19.01
CD66b$^+$ NMP Number (10^7/L)	1.23 ± 1.13	1.83 ± 1.99	1.33 ± 1.58	1.17 ± 0.76	1.44 ± 1.04	1.35 ± 1.90
CD66b$^+$ NMP % of Total (%)	0.31 ± 0.51	0.69 ± 1.11	0.44 ± 0.79	0.36 ± 0.37	0.72 ± 0.88	0.32 ± 0.66
CD14$^+$ MMP Number (10^7/L)	3.57 ± 3.61	4.37 ± 8.05	1.95 ± 1.93	2.11 ± 1.15	3.54 ± 4.20	2.88 ± 2.16
CD14$^+$ MMP % of Total (%)	0.65 ± 0.86	1.48 ± 2.76	0.71 ± 0.80	0.52 ± 0.40	1.89 ± 3.05	1.05 ± 1.22
***CD144$^+$ EMP Number (10^7/L)**	0.13 ± 0.33	0.77 ± 1.25	0.54 ± 0.74	0.73 ± 1.62	0.86 ± 0.84	0.60 ± 0.75
CD144$^+$ EMP % of Total (%)	0.01 ± 0.01	0.39 ± 0.69	0.24 ± 0.45	0.20 ± 0.39	0.57 ± 1.07	0.28 ± 0.61
***CD42b$^+$ PMP TF$^+$ Number (10^7/L)**	4.25 ± 4.97	1.44 ± 1.13	1.56 ± 0.93	3.21 ± 4.06	2.59 ± 2.21	9.51 ± 2.01
***CD66b$^+$ NMP TF$^+$ Number (10^6/L)**	4.96 ± 5.59	5.16 ± 7.00	3.82 ± 5.48	3.36 ± 2.93	5.45 ± 4.87	3.91 ± 5.24
***CD14$^+$ MMP TF$^+$ Number (10^6/L)**	11.80 ± 13.90	18.27 ± 4.92	6.36 ± 6.14	6.88 ± 5.50	15.86 ± 2.71	8.01 ± 7.34
CD144$^+$ EMP TF$^+$ Number (10^6/L)	0.39 ± 0.26	2.03 ± 3.17	1.32 ± 2.16	1.41 ± 2.81	1.54 ± 1.92	1.12 ± 1.70

Table 4.2. MP phenotype (MPs > 0.3 μm) and Tissue Factor (TF) expression results. PMP = platelet-derived MP; NMP = neutrophil-derived MP; MMP = monocyte-derived MP; EMP = endothelial cell-derived MP. '% of Total' denotes the % of each phenotype of the total MP count at that given timepoint. 'TF$^+$ % of Total PMP' denotes the % of total PMPs that were TF$^+$ - this denotation also applies to the other phenotypes. Data are presented as 'mean ± SD'. No statistically significant effects were observed in the variables presented in this table. (n = 15). * indicates that data was non-normally distributed and therefore logarithmically transformed prior to analysis.

4.5 Discussion

This study aimed to investigate the impact of a 1-hour bout of moderate intensity AE or rest on MP counts, phenotypes and pro-coagulant potential in young, healthy males over a morning period. The exercise bout induced a reduction in the proportion of platelet (immediately after exercise) and neutrophil-derived MPs (1.5 hours after exercise) that express TF that was not seen in the control trial, suggesting a reduction in their ability to induce coagulation and thrombosis.

Total MP number and diameter

Firstly, the results obtained using the NTA technique must be considered. Whilst little changes were seen in response to time or trial (this may simply be a product of the smaller cohort used for analysis, n = 5, especially given that post-hoc power calculations suggested a sample size of n = 24 would be necessary to detect changes in total MP number as assessed by NTA), the technique confirms the presence of MPs within the samples (particularly MPs < 0.3 μm) which therefore allows subsequent flow cytometry analysis in order to better characterise the particles with regards to cellular derivation, in line with recent recommendations (Szatanek et al. 2017). However, given the mean particle diameter obtained using NTA (0.15 – 0.20 μm) it seems as though the minimum detectable threshold of the flow cytometry technique (0.3 μm) would prevent the analysis of a significant proportion of the particle population. This has been shown previously and is a limitation flow cytometric MP analysis (Burton et al. 2013). However, there are several reasons why this is less problematic than initially suggested. Firstly, the low minimum detectable threshold of the nanoparticle tracking technique used here (0.05 μm) would in theory include exosomes (range in diameter of 0.04 – 0.1 μm) in the analysis. This would artificially inflate the particle concentration and also reduce the mean particle diameter independent from actual MP diameter. Secondly, no 'double confirmation' protocol was included in the nanoparticle tracking technique for the purposes of MP detection (differing to flow cytometry, which uses size and fluorescence gating). Therefore, any potential debris within the sample of a similar size to the analysed particles could not be discounted and would therefore increase the reported particle concentration (although nanoparticle tracking can incorporate fluorescence analysis, it is more arduous and less

comprehensive than flow cytometry, so it was not completed here). Therefore, whilst it is accepted that the flow cytometric analysis protocol may be excluding a significant proportion of the intended particles, flow cytometry remains the gold standard for MP analysis and still incorporates a large proportion of the reported size range of MPs (0.3 – 1.0 of the range 0.1 – 1.0 μm). Future research should aim to better align these two MP analysis techniques, as these findings support the notion that there is a need for MP analysis technique standardisation in order to better compare results from research using various analysis techniques (van der Pol et al. 2013; Van Der Pol et al. 2010; Szatanek et al. 2017). However, given that these limitations currently exist, the two techniques can be considered to be analysing slightly different populations of MPs, with MPs of 0.1 to 1.0 μm in diameter being assessed by NTA and MPs > 0.3 μm being assessed by flow cytometry. Combining the two analysis techniques provides the greatest amount of information on the entire reported MP range, albeit with slightly different reportable parameters depending on the size. However, due to logistical constraints, NTA was not completed on the entire sample (n = 15). The following discussion, reporting on the data obtained by flow cytometry, therefore concerns the MP population that is greater than 0.3 μm in diameter.

The exercise bout did not elicit significant changes in total MP (> 0.3 μm) number, as assessed by flow cytometry. As mentioned in Chapter 2, aerobic exercise-induced increased blood flow can increase shear stress (Long et al. 2004), which has been implicated in MP formation and release via modulation of cell membrane quiescence (Kim et al. 2015; Miyazaki et al. 1996; Reininger et al. 2006) due to haemodynamic cellular activation. Acute aerobic exercise may also increase cellular activation by transiently increasing catecholamine (e.g. norepinephrine) levels (Perini et al. 1989), thus increasing MP shedding by lowering membrane quiescence. However, no significant effects of the exercise bout were observed here. As with the NTA data, this may simply be a product of an insufficient sample size, as post-hoc calculations suggested that n = 36 would be required to detect a significant effect of the exercise bout. It should be noted, however, that MP characterisation based on size only (i.e. without the use of a parent cell-specific marker) is limited in its applicability to research as it is more susceptible to debris present in the sample (Dey-Hazra et al. 2010). As such, the analysis of total MPs using size gating should be considered more as a necessary step in order to better subsequently characterise MPs in

combination with a specific marker (i.e. debris within the 0.3 to 1.0 µm diameter range will trigger an event in size gating, but will not bind with an MP-specific marker and therefore will be discounted when this extra gating step is applied), rather than an outcome measure *per se*. Whilst all samples were collected, process, stored and analysed using an identical protocol, it cannot be ruled out that debris in the 0.3 – 1.0 µm diameter range influenced the total MP number in each case and therefore these results must be interpreted with caution.

Phosphatidylserine Expression

Annexin-V binds to phosphatidylserine (PS), the membrane phospholipid which is externalised during the process of MP formation and present in the MP membrane. As such, Annexin-V has been considered as a pan-cell marker for 'all MPs' in previous studies, as theoretically Annexin-V should bind to the PS which should be present in all MP membranes (although previous research has suggested that a greater amount of Annexin-V is expressed by MPs formed during apoptosis versus during cellular activation (Jimenez et al. 2003)). However, more recent research has suggested that only a small proportion of MPs bind with Annexin-V, with as many as 80% of all MPs not showing Annexin-V positivity (Connor et al. 2010). As such, this calls into question the use of Annexin-V as a pan-cell marker for all MPs. Accordingly, in this study only a very small number of MPs were found to be Annexin-V positive. Additionally, it is unsurprising that Annexin-V expression did not change in response to exercise, as it would not be expected to induce significant parent cell apoptosis (due to it being only moderate intensity (Wang and Huang 2005)) and subsequently therefore would not be expected to alter MP phospholipid membrane composition. Still, it remains unclear why such a small number of MPs would bind with Annexin-V and this should be further investigated in order to determine better pan-cell markers which will ultimately lead to better characterisation on MP populations.

MP Phenotypes and Proportions

The number and proportions of each MP phenotype were not altered by exercise. Firstly, as with total MP number assessed by NTA, this could be a product of an insufficient sample size, as post-hoc power calculations suggested a sample size of n = 40 would be necessary to elicit a significant interaction effect

in MP phenotype numbers. As discussed in Chapter 2, the results of studies that investigated the impact of aerobic exercise on MP phenotype counts are conflicting (Chaar et al. 2011; Guiraud et al. 2013; Wahl et al. 2014) and may potentially depend on training status. As the participants in this study were relatively fit from an aerobic capacity perspective (mean VO_2 max of 54.9 $mL \cdot kg \cdot min^{-1}$) the results would be expected to more closely mimic those found in trained populations, which primarily found either no change (Guiraud et al. 2013) or a reduction (Wahl et al. 2014) in endothelial cell-derived MPs following an acute bout of aerobic exercise (varying in 55-100% peak power output). As explained in Chapter 2, the increased blood demand of the working muscles during aerobic exercise elicits increased blood flow which in turn increases shear stress, which causes haemodynamic activation of leukocytes, platelets and endothelial cells, ultimately leading to reduced membrane quiescence and increased MP formation (Kim et al. 2015). However, in aerobically trained individuals improved endothelial function and increased NO availability (Green et al. 2004) may dampen the aerobic exercise-induced increase in shear stress and thus prevent increased endothelial cell-derived MP formation. However, it is unclear how this adaptation effect would affect other MP phenotypes. Similarly, the aerobic exercise-induced increase in catecholamine release would also be expected to activate leukocyte and thus increase MP release (Perini et al. 1989), however it is unclear why this did not occur in this study. Additionally, a recent study found that, following 20 min of moderate intensity (65% of VO_2 max) AE, a significant reduction in the number of $CD62E^+$ (endothelial-derived) MPs was observed in female participants only, with no change in the male participants (Shill et al. 2018). This suggests a sex difference with regards to MP responses to AE and provides a potential explanation as to why no changes were seen in MP number in this all-male study sample. A possible reason behind this sex-dependent difference is oestrogen levels. Oestrogen levels have been shown to impact MP formation from different cell types (low oestrogen levels are associated with accelerated MP formation) (Jayachandran et al. 2009). However, it is currently unclear if this relationship would impact the MP exercise response in females, as greater oestrogen levels seen in female participants would in theory reduce circulating MP levels and thus make it less likely to see an exercise-induced reduction. Low testosterone levels may explain the sex disparity, as low testosterone levels have been associated with increased circulating concentrations of endothelial cell-

derived MPs (though this was only investigated in men with erectile dysfunction) (Omar et al. 2017). Further research should be completed to characterise the sex-dependent MP response to aerobic exercise.

Tissue Factor Expression

MPs have been proposed to provide a catalytic surface within the circulation for the initiation and promotion of coagulation and thrombosis (Ando et al. 2002). Additionally, TF expressed on the surface of MPs promotes coagulation via stimulation of the coagulation factor FVII/FVIIa and P-Selectin (an adhesion molecule localised within platelet membranes) (Polgar et al. 2005) and thus elicits platelet aggregation (Del Conde et al. 2005). Therefore, increased TF^+ MP numbers are postulated to increase cardiovascular risk via increased risk of thrombotic events, which are the primary cause for many cardiac events such as myocardial infarction or stroke (Ridker et al. 1994; Ridker et al. 1994) . In this study, whilst the absolute number of TF^+ MPs was unaffected by exercise, the proportion of TF^+ platelet and neutrophil-derived MPs were significantly reduced by the acute exercise bout whilst remaining unaffected in the control trial. Acute AE and regular aerobic training have previously been shown to elicit favourable changes in thrombotic potential via mechanisms such as reduced fibrinogen concentration (Wosornu et al. 1992; Zanettini et al. 1997) and increased tissue-type plasminogen-activator resulting in stimulated fibrinolysis and thrombolysis (Smith et al. 2003). To the author's knowledge, this is the first study to propose another mechanism, namely reduced proportion of TF^+ MPs in response to moderate intensity acute AE. However, it remains to be seen whether the absolute number of TF^+ MPs or the relative proportions are more significant with regards to coagulation and thrombotic potential. Similarly, the nature of this relationship in response to regular AE training is unknown, as well as whether or not patient populations at greater cardiovascular risk would respond in a similar manner. Further research is required to investigate the effect of regular exercise training in at-risk populations for thrombotic events to determine if reduced MP TF expression may be another mechanism via which CV risk can be decreased.

Lastly, this finding points to the importance of assessing MP functional characteristics rather than just absolute phenotype numbers. Further research should include measures of MP functional characteristics (or even MP function measured on an *in vitro* scale) and how they respond to exercise as this may be more meaningful with regards to health than simple enumeration. For instance, prothrombin assays have been used in previous research to quantify the thrombotic potential of MPs (Owens and Mackman 2011). However, as the analysis of MPs is time and cost-intensive, the MP analyses in this study (and in the following studies included in this thesis) were focussed on MP phenotype characterisation and thrombotic potential (using TF expression). Further functional assays were considered to be beyond the scope of the analyses completed in this thesis.

Limitations

This study has several limitations which must be considered in order to appropriately interpret the findings. Firstly, when considering the post-hoc sample size calculations, the sample size investigated here (n = 5 for NTA and n = 15 for flow cytometry) was insufficient to detect significant changes in response to the exercise bout in total MP number or phenotype-specific MP number. Another limitation is the reported mean MP diameter obtained by NTA, and the subsequent discrepancy with the size thresholds used in the flow cytometry technique. However, there are certain limitations to NTA when compared to flow cytometry. Firstly, as described earlier, NTA does not provide the level of detail with regards to phenotypic characterisation (e.g. multiple concurrent markers) that is provided by the flow cytometry protocol. Secondly, subjective alterations in the instrument settings (e.g. camera level and gain) can affect quantification of MPs using NTA. Current guidelines propose that flow cytometry remains the most commonly used technique with the best capability to determine the cellular origin of MPs (Cointe et al. 2017). Therefore, the flow cytometry technique implemented here is considered to be robust and the most appropriate method to answer the proposed research question. However, combining this technique with NTA allows the examination of the entire reported diameter range of MPs (though this technique was unavailable for use for analysis in Study 2, 3 and 4 and as such has only been completed on Study 1). However, another obvious limitation is the large coefficient of variation of

the flow cytometric analysis technique (13.88%). This increases the chance of missing a potentially significant change (i.e. Type 1 error).

Other limitations concerning the study protocol must also be considered. The standardised meal was consumed after exercise and may have influenced MP kinetics; this makes it difficult to comment on the MP response during exercise recovery only as it was not assessed in isolation (though any meal-dependent effects would be identical in both trials). However, as the participants arrived at the laboratory in in a fasted state, it was considered to be more ecologically valid to provide a post-exercise meal rather than continue fasting, as this would more closely resemble the format of AE completed outside of the laboratory setting (though it is acknowledged that fasted exercise may also reduce ecological validity). Additionally, due to the time-intensive nature and relative expense of analysing MPs via flow cytometry, only the pro-thrombotic potential (TF expression) of MPs was assessed from a functional perspective, and no further functional assays were completed. As discussed in Chapter 2, MPs possess the ability to promote a number of other pathophysiological processes, including influencing systemic inflammation, promoting oxidative stress and impairing endothelial function. Therefore, to fully investigate the impact of acute AE on MP functionality, these relationships must also be considered. However, as mentioned above, the labour-intensive nature of MP analysis prevented the inclusion of functional MP assays in this thesis. Lastly, there are other sources of MPs that were not assessed in this study, given that almost all types of mammalian cells can produce and release MPs (Wu et al. 2013). As such, other MP derivations may have been influenced by exercise. However, the phenotypes assessed in this study are the most commonly assessed in previous literature and are therefore well characterised, making them easier to detect via flow cytometry and allowing a more direct comparison with previous results.

Conclusions, implications and further research

In summary, a 1-hour bout of moderate intensity AE did not elicit changes in total MP number, phenotype-specific number or proportions relative to rest, however did reduce the proportion of platelet- and neutrophil-derived MPs that positively expressed TF. This may provide another mechanism

via which AE is able to reduce cardiovascular risk – i.e. through reduced stimulation of coagulation and thrombosis. This effect therefore requires more investigation in clinical populations, particularly those who are at greater risk of cardiovascular disease. The assessment of the pro-thrombotic potential of MPs in response to aerobic exercise training in clinical populations could provide another method to assess intervention efficacy, and ultimately could be used to tailor individual exercise programmes in order to greater reduce cardiovascular disease risk. If looking to further investigate the impact of acute exercise on MP phenotype numbers, future studies should employ larger sample sizes in order to detect significant results.

As mentioned previously, though previous efforts has been made to investigate the response of MP numbers to acute exercise in clinical populations (Highton et al. 2018), only one recent study (to the author's knowledge) has attempted to investigate the functional significance of the response (Martin et al. 2018) – this is an avenue that should be investigated further. As such, the following studies included in this thesis will investigate whether or not this acute effect influences resting MP TF expression following regular aerobic exercise in haemodialysis patients (Study 2) and whether or not this acute effect is observed in other renal patient populations (Study 4).

Chapter 5

Study 2

The effects of intradialytic cycling on markers of systemic inflammation in haemodialysis patients

5.1 Abstract

End stage renal disease (ESRD) patients receiving haemodialysis (HD) treatment display increased circulating markers of chronic systemic inflammation and altered circulating microparticle (MP) numbers and characteristics. Regular aerobic exercise may help to alleviate or improve these pathophysiologic mechanisms, however HD patients report significant perceived barriers to traditional exercise participation, such as safety concerns or lack of free time and as such may not regularly participate in interdialytic exercise programmes. Intradialytic cycling (IDC) circumnavigates these issues as it is completed during HD treatment in the presence of HD nurses to ensure patient safety. The aim of this study was to investigate the impact of 6-months of thrice-weekly moderate intensity IDC on markers of systemic inflammation and circulating MPs in HD patients, in comparison with a usual care HD control group.

Forty HD patients were randomised to IDC (n = 20; age: 51.4 ± 18.1 years; body mass: 77.6 ± 18.3 kg; 12 males; 'mean ± SD') or usual care control (n = 20; age: 56.8 ± 14.0 years; body mass: 80.5 ± 26.5 kg; 12 males). The IDC group cycled for 40 minutes, thrice weekly during every HD treatment for 6 months at an RPE of 12-14 – mean session characteristics were recorded. Physical function tests (sit-to-stand and shuttle walk tests) and pre-dialysis resting venous blood samples were completed at 0, 3 and 6 months. In venous blood, circulating cytokines (IL-2, IL-6, IL-10, IL-17a, TNF-α), chemokines (IL-8, MCP-1, MIG, IP-10) and microparticle (MP) phenotypes (platelet, neutrophil, monocyte or endothelial cell-derived) and prothrombotic potential (tissue factor (TF) expression) were quantified using flow cytometry analysis protocols. Statistical analysis was completed using IBM SPSS version 23.

Effect sizes are presented as η^2 (0.2 = small, 0.5 = moderate, 0.8 = large, Cohen, 1988). Groups were well matched at baseline for demographic and clinical characteristics. Despite significant progression in cycling resistance ($p < .001$, $\eta^2 = .288$) and high exercise compliance (82%), mean session power (21 Watts), estimated distance and estimated energy expenditure remained unchanged from pre to post-intervention ($p \geq .368$, $\eta^2 \leq .055$). Similarly, no improvement was reported in sit-to-stand or shuttle walk test performance in either group ($p \geq .229$, $\eta^2 \leq .048$).

Circulating TNF-α concentration reduced from baseline to final in the exercise group only ($p = .001$, $\eta^2 = .272$), though no changes were observed in IL-6 or IL-10 and IL-17a and IL-2 were largely undetectable. No significant effects were observed in measured chemokines or MP phenotype numbers or characteristics.

The lack of change in mean exercise session characteristics and by extension physical function performance may be a result of the relatively low exercise intensity, despite exercise compliance being high. The reduction in circulating TNF-α in the IDC group is encouraging and suggests an overall anti-inflammatory effect of exercise. The lack of changes in all other inflammatory markers suggests that the exercise did not elicit aberrant immune activation. Further research should include larger sample sizes (as this study was restricted to a cohort of 40 patients due to the time-intensive nature of the laboratory analysis). Additionally, it may be pertinent to identify those patients that will benefit most from IDC interventions, for instance by completing a larger IDC study that can stratify patients based on relevant clinical characteristics (e.g. primary diagnosis, HD vintage, comorbidities) in order to create more targeted exercise programmes.

5.2 Introduction

Haemodialysis (HD) patients display elevations in both cardiovascular and all-cause mortality compared to the general population, which can only be partly explained by traditional risk factors (Longenecker 2002). Infection closely follows as the second leading cause of death in this patient population (24% CVD-related deaths versus 20% infection related deaths in 2014) (Steenkamp et al. 2018).

There are many possible causes for these relationships in HD patients. Compared to the general sedentary population, they display reduced levels of habitual physical activity (Johansen et al. 2000), worsened body composition (increased fat mass, decreased lean mass) (Johansen et al. 2003) and elevated markers of chronic systemic inflammation (Chiu et al. 2009), caused by both an increased basal level of pro-inflammatory cytokines such as IL-6, TNF-α and IL-17 (Rysz, Banach, and Cialkowska-rysz 2006; Zhang et al. 2010) and a reduced basal level of anti-inflammatory cytokines such as IL-10 and TGF-β (Zhang et al. 2010).

This systemic inflammatory environment can be attributed to a chronic ligation and thus aberrant activation of monocytes and T lymphocytes by uraemic toxins, which leads to persistent yet unnecessary cytokine secretion (Hauser et al. 2008; Sester et al. 2000), as well as immune cell subset distribution alterations (e.g. increased proportion of pro-inflammatory $CD16^+$ monocytes, reduction in $CD4^+/CD8^+$ lymphocyte ratio) (Heine et al. 2012; Zhang et al. 2010) and ultimately impaired cellular immune function (Girndt et al. 1999). Other possible causes for chronic immune over-activation and thus systemic inflammation include acidosis (Lardner 2001) due to a reduction in renal buffering capacity, oxidative stress (Libetta et al. 2011) elicited by increased ROS production and decreased NO availability, or endotoxin exposure due to blood contact with the extracorporeal circuit (Libetta et al. 2011), dialysis membrane (Wanner et al. 2004) or dialysate. The consequence of this chronic immune over-activation is a persistent and pathophysiological secretion of pro-inflammatory cytokines, resulting in a systemic pro-inflammatory environment (Stenvinkel and Alvestrand 2002). In ESRD patients, elevations in pro-inflammatory cytokines have been associated with increased aortic stiffness (Desjardins et al. 2017), elevated cardiovascular disease risk (Wanner et al. 2002) and elevated cardiovascular and all-cause mortality (Barreto et al. 2010). As such, a dampening of the pro-

inflammatory milieu in ESRD patients represents an important target for morbidity and mortality reduction.

Chemokines are a subset of cytokines that promote the chemotaxis of specific leukocytes and help to co-ordinate the immune response by facilitating the migration of cells to the site of infection or injury (Griffith, Sokol, and Luster 2014). However, similarly to cytokines, chemokines can exhibit a detrimental effect when allowed to proceed unregulated. Unregulated chemokine secretion can result in unnecessary migration and extravasation of leukocytes into the vasculature, thus driving atherosclerosis by increasing the pool of resident cells within the intima and media and creating a local pro-inflammatory environment (van der Vorst, Döring, and Weber 2015). Due to the chronic immune system activation in HD patients as explained above, this population displays elevated chemokine concentrations (e.g. monocyte chemotactic protein-1 (MCP-1)) which have been positively associated with elevated systemic inflammation (CRP) (Papayianni et al. 2002; Papayianni et al. 2003). The previously described inflammatory activation of chemokines has been shown to contribute to atherosclerosis in ESRD patients by increasing foam cell formation within the vasculature (Hu et al. 2016). As such, a reduction in circulating chemokine concentrations would be expected to reduce cardiovascular disease risk in ESRD patients (Dusi et al. 2016).

Microparticles (MPs) are small (0.1-1.0 μm) extracellular vesicles shed upon cellular activation or apoptosis and can act as biomarkers for inflammation and leukocyte and endothelial dysfunction. MPs can also drive inflammation, oxidative stress and thrombosis through several pathways (e.g. promoting leukocyte cytokine secretion, reducing endothelial NO release, or increasing TF bioavailability to drive thrombus formation) (Ando et al. 2002; Piccin et al. 2007; Polgar et al. 2005). A single dialysis treatment session has been shown to increase the circulating concentration of platelet and neutrophil-derived MPs, which has been attributed to the acute pro-inflammatory effect of the HD membrane and dialysate, resulting in increased leukocyte activation (Daniel et al. 2006). Consequentially, resting circulating MP levels are elevated in comparison to the healthy population, and MPs from HD patients also display elevated thrombotic potential compared to the healthy population (Burton et al. 2013). As such, resting circulating MP levels may be predictive of mortality in this population (Amabile et al.

2012). Therefore, any intervention which can ameliorate the increase in resting MP levels or reduce the MP pro-thrombotic potential seen in HD patients would be expected to reduce morbidity and mortality in this patient group.

A therapeutic strategy that has the potential to positively influence all the detriments mentioned above is aerobic exercise. The beneficial effects of regular, moderate intensity aerobic exercise participation on the cardiovascular system are well documented in both the general population (Shephard and Balady 1999) and the HD population (Deligiannis et al. 1999; Mustata et al. 2004). In the CKD population, traditional aerobic exercise training has proven effective in improving body composition (reducing visceral fat mass) (Baria et al. 2014), improving functional capacity (Painter et al. 2000) and dampening systemic inflammation (reduction in IL-6/IL-10 ratio) (Viana et al. 2014). However, HD patients experience significant perceived barriers to traditional aerobic exercise training, including perceived lack of time, concerns over safety and lack of exercise knowledge and opportunities (Delgado and Johansen 2012). Intradialytic cycling (IDC) circumnavigates these issues, as it is completed during HD treatment and thus requires no extra time sacrifice, and is commonly completed either under the supervision exercise professionals or in the presence of HD nursing staff and clinicians, mitigating potential patient safety concerns. IDC training has been shown to reduce arterial stiffness and cardiovascular risk (Toussaint et al. 2008), enhance physical performance (Parsons, Toffelmire, and King-VanVlack 2006), and increase health-related quality of life (Ouzouni et al. 2009).

In HD patients, an acute bout of moderate intensity IDC has also been shown to exert an anti-inflammatory influence (decreased IFN-γ, IL-6 and TNF-α release and increased IL-10 release) (Peres et al. 2015; Wong et al. 2017) without negatively affecting monocyte subset distribution or neutrophil function (Dungey et al. 2015). However, methodological limitations exist in the current body of literature, for instance a lack of pre-dialysis baseline samples (Dungey et al. 2017), inappropriate control groups (Gołębiowski et al. 2012; Zaluska et al. 2002) or inadequate sample sizes (Toussaint et al. 2008). As listed in Table 2.7, several studies have shown that regular moderate intensity IDC can either reduce or prevent an increase in circulating inflammatory mediators (CRP, IL-6) (Afshar et al. 2010, 2011; Liao et al. 2016; Zaluska et al. 2002). However, these studies did not include measures of other cytokines that

are elevated in HD patients, such as TNF-α or IL-17a (Rysz et al. 2006; Zhang et al. 2010) or any anti-inflammatory cytokines (e.g. IL-10) which may be increased by regular aerobic exercise training (Goldhammer et al. 2005). However, as is also listed in Table 2.7, a number of studies investigating regular IDC training found no impact on inflammatory markers (Dungey et al. 2017; Gołębiowski et al. 2012; Toussaint et al. 2008; Wilund et al. 2010) – the cause for this disparity in the previous literature is unclear. Additionally, there is no research in the current body of literature that has investigated how regular IDC training affects chemokine (e.g. MCP-1, IL-8, monokine induced by gamma interferon (MIG)) concentrations, and only one study has investigated the impact of IDC on MPs (Martin et al. 2018), and this was on an acute basis only and suggested a possible ROS-mediated anti-inflammatory effect. In the general population, traditional regular aerobic exercise training has promoted favourable changes in these markers (reduced circulating MP and chemokine levels) (Babbitt et al. 2013; Trøseid et al. 2004) and therefore their responses to IDC in the HD population require further investigation. This will help to elucidate the possible mechanisms linking aerobic exercise to vascular health in the HD population. Therefore, the aim of this study was to investigate how 6 months of thrice-weekly (every HD session), moderate intensity IDC affects the resting concentrations of pro- and anti-inflammatory cytokines, chemokines and MPs. It is hypothesised that regular intradialytic cycling will dampen systemic inflammation (i.e. reduce resting pro-inflammatory cytokine levels whilst increasing resting anti-inflammatory cytokine levels), reduce chemokine concentration and reduce the concentration and pro-thrombotic potential of circulating MPs.

5.3 Methods

Ethics

This study was completed as a subset of the multi-site 'CYCLE-HD' study (protocol published by Graham-Brown et al. 2016) (CYCLE-HD: *Improving cardiovascular health in dialysis patients using a structured programme of exercise,* ISRCTN registration number 1129707), funded by a grant from the National Institute of Health Research (NIHR) and sponsored by the University Hospitals of Leicester (UHL) Trust. All aspects of this study were given favourable opinion by the NHS Research Ethical Committee (ref. 14/EM/1190) and the University Hospitals of Leicester Ethical Committee (UHL 164347).

Recruitment

Patients were deemed fit to participate in the study by a Consultant Nephrologist prior to giving written informed consent. Patients were approached during their routine haemodialysis sessions, provided they were deemed eligible by the consultant and violated none of the exclusion criteria. Patients had the study protocol thoroughly explained to them, had the opportunity to ask questions if necessary and were given at least 48 hours to consider their participation. Willing patients then provided written informed consent.

Inclusion criteria

1. Prevalent haemodialysis patient (> 3 months)
2. Aged 18 years or older
3. Able and willing to give informed consent

Exclusion criteria

1. Unable to participate in current exercise program due to perceived physical or psychological barriers
2. Unable to undergo MRI scanning (metal implants / prostheses, claustrophobia etc.)
3. Unfit to undertake exercise according to the American College of Sports Medicine guidelines

4. contraindications to exercise testing that include:
    a. Recent significant change in resting ECG that suggests significant ischaemia, recent myocardial infarction (2 weeks) or other acute cardiac event;
    b. Unstable angina;
    c. Uncontrolled cardiac dysrhythmias causing symptoms or haemodynamic compromise;
    d. Symptomatic severe aortic stenosis;
    e. Uncontrolled symptomatic heart failure;
    f. Acute pulmonary embolus or pulmonary infarction;
    g. Acute myocarditis or pericarditis;
    h. Suspected or known dissecting aneurysm;
    i. Acute systemic infection, accompanied by fever, body aches or swollen lymph glands.
5. Age <18 years
6. Unable or unwilling to give informed consent

Randomisation

As explained in Chapter 2, HD patients receiving conventional unit-based HD therapy in the UK dialyse in one of two cohorts: either on a Monday, Wednesday and Friday or on a Tuesday, Thursday and Saturday. Based on previous experience from a pilot exercise programme completed by our research team, the introduction of IDC creates a more positive attitude towards exercise in the entire cohort, not just those individuals undertaking exercise. This precludes the use of traditional randomisation as that would allow for potential contamination effects (i.e. a control patient receiving dialysis next to a patient completing IDC will be indirectly influenced by the intervention). Therefore, one dialysis cohort was randomised to the intradialytic exercise intervention and the other was randomised to non-intervention control at each HD unit. Therefore, there was no interaction between control and exercise patients and no contamination effect was possible. The patients were not informed of this randomisation protocol and so were unaware of their allocation when they consented to their participation. Their study allocation was revealed to them after the completion of the baseline assessments – the point at which

the intervention would begin in the cycling group. This 'cluster' randomisation protocol was modelled by the Robertson Biostatics Centre at the Glasgow Clinical Trials Unit and was peer reviewed by the NIHR.

Study design

The intervention implemented in this study was of 7 months in duration. The first month of IDC was utilised as a familiarisation and 'run-in' period, during which the patients could become familiarised with the bike and slowly increase their exercise duration. The following 6 months comprised the structured exercise programme. Baseline assessments were completed at 0 months, interim assessments were completed at 4 months (i.e. after 3 months of structured exercise or control) and final assessments were completed at 7 months. This protocol along with the accompanying outcome measures (including all those completed in the CYCLE-HD study and not just those included in this chapter) is displayed in the schematic in Appendix 5.

Cohort selection procedure

Due to the large number of patients recruited into the entire CYCLE-HD study, it was necessary to select a smaller cohort of patients for inclusion in this thesis to allow for the feasibility of completing all of the required laboratory analyses. As such, a cohort was selected that comprised all the eligible patients that had completed the intervention at the time of analysis, as waiting for the entire study to be completed would have not allowed enough time for analysis and would have presented too many patients to be feasible with regards to the scope of the time and cost-intensive laboratory analysis presented in this thesis. At the time of cohort selection, the total number of patients consented into the study was 128. Of these, 64 had either dropped out or not yet finished the intervention and so were excluded, leaving 64 completed patients. From these 64, those who were missing blood samples (i.e. missing an entire timepoint) (n = 8), taking immunosuppressive medication (n = 6), had received a blood transfusion during the study (n = 1) or had significant missing data other than blood samples that would preclude potential analyses (n = 9) were excluded. This left 40 total patients, 20 from each of the control and exercise groups who were included in this cohort and the associated analyses. As this was more of an

opportunistic cohort selection procedure, a power calculation was not completed in order to arrive at this sample size. This selection protocol is demonstrated in the consort diagram in Figure 5.1.

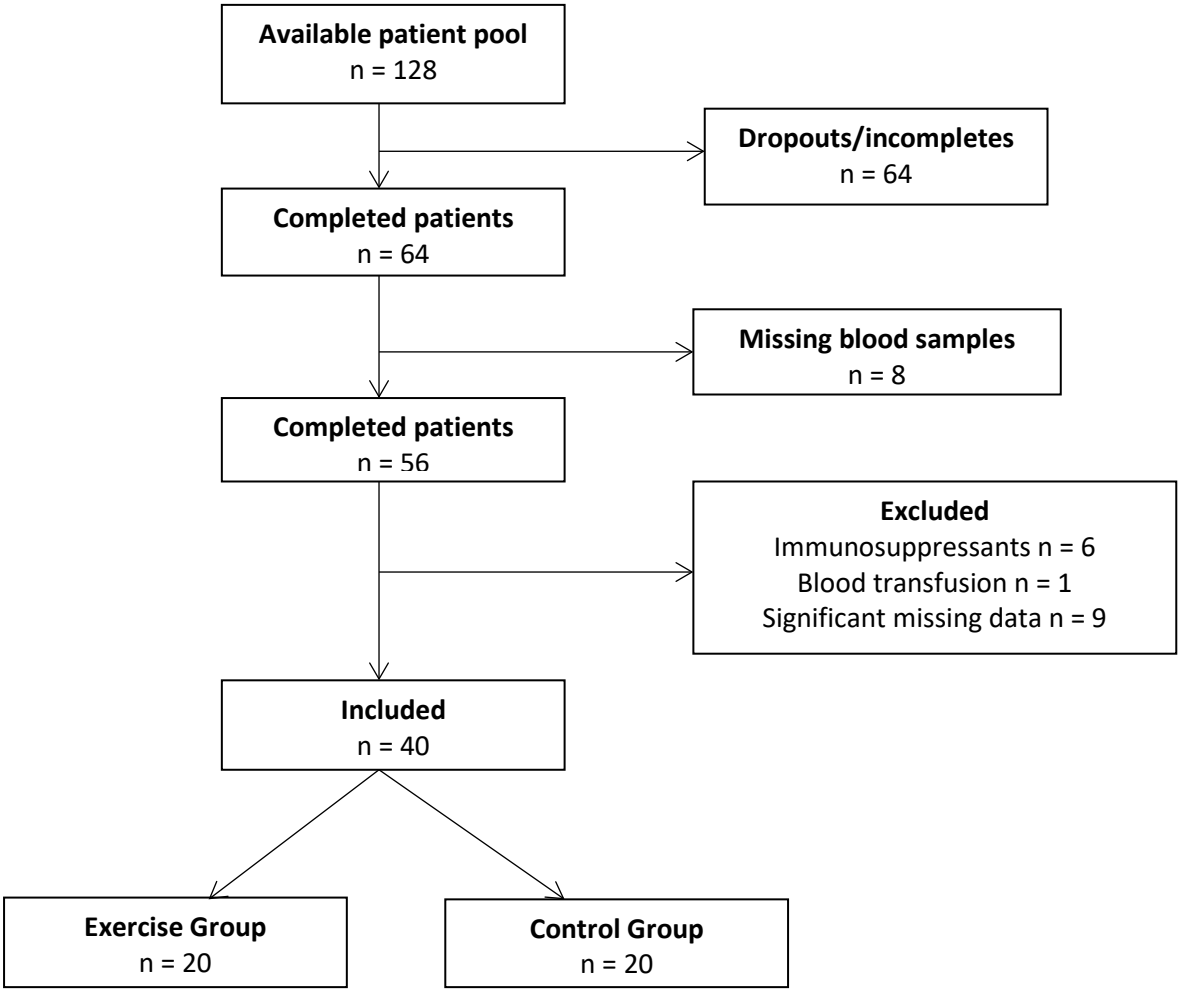

Figure 5.1. Cohort selection procedure flow diagram from the whole CYCLE-HD data set.

Intradialytic exercise programme

All patients allocated to the exercise group in their respective HD unit completed IDC during their routine HD treatment sessions, three times per week. IDC was completed on a specifically designed cycle ergometer (Letto 2, Motomed, Reck, Germany) that allows the completion of exercise whilst remaining in the semi-recumbent dialysis chair. IDC was not offered within the first 30 minutes of each HD session to ensure that the treatment was working correctly before exercise initiation. Similarly, to prevent any complications regarding the simultaneous occurrence of both post-exercise and post/late-

HD hypotension, IDC was always completed before the last 90 minutes of HD in line with previous suggestions (Moore et al. 1998).

The electronically-braked ergometers provided a range of gears from 1 to 20. Patients were encouraged to precede each IDC bout with a 5-minute warm-up period and follow it with a 5-minute cool-down of self-selected intensity. Patients initially cycled for a short duration and on a low gear to ensure they were confident and comfortable with the ergometer during the 1-month run-in period. They were then encouraged to increase their exercise duration to 30 minutes (40 in total including warm-up and cool-down), as well as increase their gear during this 30 minutes to ensure adequate exercise intensity. Exercise intensity was assessed using a rating of perceived exertion (RPE) scale (Borg, 1973). Patients were educated on how to use this scale prior to programme initiation and were encouraged to exercise at a gear that represented 'somewhat hard' exercise (RPE 12-14), whilst maintaining an average speed of 60-70rpm. Duration and gear progression was encouraged in order to maintain an RPE rating of 12-14. An image of HD patients using the bike is displayed in Figure 5.2.

Patients did not exercise if they were deemed temporarily unsuitable by a member of clinical staff. Exercise was not completed if the patient was severely hyper or hypotensive (systolic blood pressure above 190mmHg or below 90mmHg), temporarily anaemic (haemoglobin < 8 $g \cdot dL^{-1}$) or had significant fluid accumulation in the previous interdialytic period (ultrafiltration goal of over 3.0L). These measures were recorded by the clinical staff before dialysis, and were checked by a researcher prior to exercise initiation. Patients were also entitled to refuse exercise; however, all patients were encouraged to exercise every session provided they were physically capable of doing so and there were no contraindications.

The cycle ergometers provided measures of average power (W), average speed (rpm), estimated distance travelled (km) and estimated energy expenditure (kcal). These values were recorded after every IDC bout along with RPE and gear ratings and were used to monitor progress.

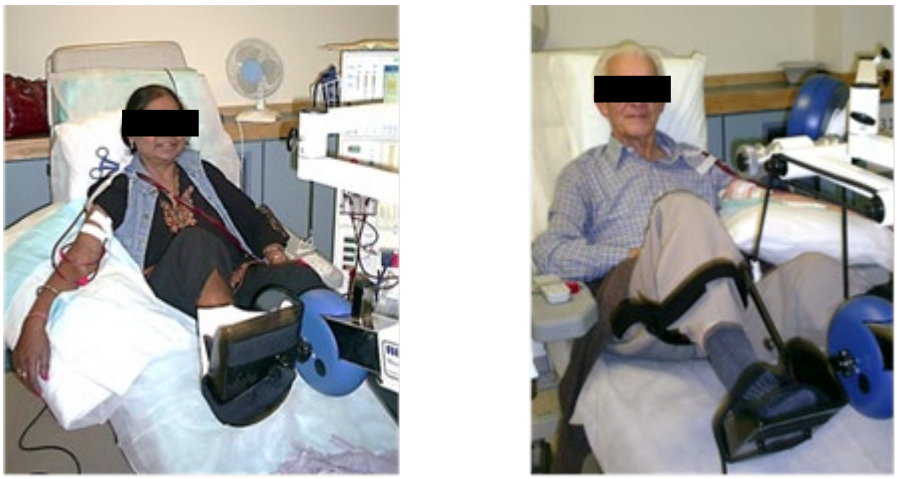

Figure 5.2. Demonstration of the use of the specifically designed cycle ergometer, completed whilst undergoing treatment in a regular dialysis chair.

Blood sampling and storage

Resting blood samples were taken on a dialysis day, prior to initiation of treatment and not following the 'long-break' in HD treatment over the weekend (i.e. not on a Monday or Tuesday). Venous blood (30mL in total) was drawn from the patient's vascular access prior to them being connected to the dialysis machine, directly into monovettes containing either K_3EDTA (1.6 mg/ml blood) or sodium citrate (0.106 mol/L) as anticoagulants (Sarstedt, Nümbrecht, Germany). Venous blood samples were processed and stored for future analysis (described in detail in General Methods - Chapter 3, briefly: K_3EDTA-treated blood was centrifuged at 2,500g for 10 minutes at 4°C, supernatant (plasma) was separated and stored at -80°C; sodium citrate-treated blood was centrifuged at 2,500g for 15 minutes at 20°C, after which the top 90% (plasma supernatant) was removed and centrifuged again at 2,500g for 15 minutes at 20°C – the supernatant (platelet-free plasma) was then removed and stored at -80°C). Blood samples were taken at 0, 4 and 7 months (baseline, interim and final assessment timepoints, respectively).

Physical function tests

Muscular power and muscular endurance were assessed using the sit-to-stand (STS) 5 and 60, respectively (Bohannon 1995). The STS5 requires the patient to stand from a seated position until fully upright and then return to the seated position – this is completed 5 times as fast as possible and is a surrogate measure of the muscular power of the lower limbs (Lindemann et al. 2003). The STS60

requires the patient to complete this same movement as many times as possible in 60 seconds and is a surrogate measure of muscular strength and endurance (Ritchie et al. 2005).

The ISWT (described in chapter 3) requires the participant to walk up and down a level 10m course in time with a progressively quickening beep until either the pace becomes too quick or volitional exhaustion is reached – the total distance achieved is a surrogate measure of VO_2 peak (Singh et al. 1992). These assessments were completed at 0, 4 and 7 months.

Clinical data and routine blood tests

Relevant clinical data and routine blood test results were extracted from medical records as described in Chapter 3. Briefly, this information included comorbidities, medications (extracted at 0 and 7 months) and anthropometrics (extracted at 0, 4 and 7 months). Blood results were all extracted at 0, 4 and 7 months and included leukocyte numbers, renal profiles, blood lipid levels and measures of dialysis adequacy.

Laboratory sample analysis

Plasma concentrations of circulating cytokines (IL-2, IL-6, IL-10, IL-17a, TNF-α) and chemokines (IL-8, MIG, MCP-1, IP-10) were multiplexed and analysed using a cytometric bead array technique in combination with a BD Accuri C6 Flow Cytometer, as described previously (Chapter 3). All cytokines were measured using an enhanced sensitivity flex set approach (Enhanced master buffer kit combined with kits for individual cytokines (human anti-IL-2, human anti-IL-6, human anti-IL-10, human anti-IL17a, human anti-TNF-α – all fluorescing on FL2)) (BD Biosciences, Oxford, UK). As mentioned in Chapter 3, any sample that fell below the minimum detectable threshold was omitted from the statistical analysis and is not presented. The number of included/omitted samples is presented within each variable in the results section. All chemokines were analysed using a pre-set kit (anti-human CXCL8/IL-8, anti-human CXCL9/MIG, anti-human CCL2/MCP-1, anti-human CXCL10/IP-10 – all fluorescing on FL2) (BD Biosciences, Oxford, UK).

Concentrations of circulating microparticles were analysed as described earlier (Chapter 3). Briefly, MPs were labelled using specific antibodies conjugated to fluorochromes, and enumerated via flow

cytometry (BD Accuri C6). MPs were characterised based on size, Annexin-V expression (anti-AnV FITC), TF (CD142) expression (anti-CD142 APC) and their cellular origin, i.e. platelet ($CD42b^+$) (anti-CD42b PE), neutrophil ($CD66b^+$) (anti-CD66b PE), monocyte ($CD14^+$) (anti-CD14 PE) or endothelial cell ($CD144^+$) (anti-CD144 PE) derived MPs.

Statistical analysis

Any non-normally distributed data was logarithmically transformed prior to analysis. All data was either normally distributed or successfully logarithmically transformed prior to analysis - this information is presented within the results presentation for each variable. Whilst testing for baseline imbalances in an appropriately randomised trial is unnecessary and in theory futile (Lydersen 2015), this study was completed on a cohort of patients selected from the full randomised data set and as such was subjected to a secondary, different 'randomisation' procedure during the cohort selection. Therefore, to investigate the impact of this secondary selection on condition randomisation efficacy, comparison of baseline characteristics was completed using independent samples t-tests. Where the assumption of equality of variances was violated, the adjusted p value has been presented. Exercise progression data was analysed using a within-measures one-way ANOVA, as there was no data for the control condition - any significant findings were elucidated using the Bonferroni post-hoc correction. The impact of the exercise intervention in comparison to the control condition was assessed using a mixed design ANOVA, allowing the detection of time*group interaction effects, if they were present. When Mauchly's Test of Sphericity was violated, the Greenhouse-Geisser p value was reported. Partial eta squared (η^2) was used to estimate effect sizes for all ANOVAs. Effect sizes were classified as small (0.2), medium (0.5) and large (0.8) (Cohen 1988). If a time*group effect was detected, an independent samples t-test was completed between groups at each time-point to elucidate the effect. For all analyses, $p < .05$ was considered statistically significant. All descriptive data are presented as 'mean ± standard deviation' whilst all outcome data is reported as 'mean ± standard error of the mean' when presented in a figure, and 'mean ± SD' when presented in a table or discussed in the text. All statistical analysis was completed on IBM SPSS version 23.0 (Chicago, Illinois) and all graphs were produced on GraphPad Prism (v.6 GraphPad Software Inc., CA, USA).

5.5 Results

Participants

As explained in section 4.4, the cohort selection procedure resulted in 40 patients being included in these analyses (exercise n = 20, control n = 20). All patient demographic and clinical data are presented in Table 5.1. Groups were well matched for demographic and anthropometric measures, as well as primary diagnosis, HD vintage, comorbidities and the majority of routine clinical blood results. However, the control condition exhibited a significantly higher haemoglobin value ($p = .047$), and significantly lower albumin ($p = .014$) and phosphate ($p = .047$) values.

As well as showing no difference between groups at baseline, neither body mass nor BMI presented a significant time*group interaction effect ($p \geq .837$, $\eta^2 \leq .005$). The same can be said for both systolic and diastolic resting blood pressure ($p \geq .549$, $\eta^2 \leq .016$).

Similarly, the trial had no effect on the number or dose of medications being taken in either group (recorded at baseline and final only). The number of anti-hypertensives, Eprex dose, iron dose and number of phosphate binders displayed no time*group interaction effect ($p \geq .233$, $\eta^2 \leq .037$).

Metabolic and nutritional markers also remained unchanged throughout the trial. Total cholesterol, triglycerides, albumin and HbA1C all exhibited no time*group interaction effect ($p \geq .214$, $\eta^2 \leq .053$).

Leukocyte counts also showed no changes in response to the trial. Numbers of neutrophils, lymphocytes and monocytes showed no significant time*group interaction effect ($p \geq .174$, $\eta^2 \leq .068$). Consequentially, the total white blood cell count displayed no significant time*group interaction effect ($p = .447$, $\eta^2 = .021$).

Exercise compliance and progression

Exercise compliance within the cycling condition was high – 82% of sessions were completed during the 6-month intervention. Of the total number of missed sessions (n = 268), reasons for missed sessions included; patient declined without giving a reason (41%); patient reported being unwell (26%); patient missed their usual HD slot (13%); patient reported pain (12%); problems with the HD machine or

Timepoint	Baseline		Interim		Final	
Group	**Control (n = 20)**	**Cycling (n = 20)**	**Control (n = 20)**	**Cycling (n = 20)**	**Control (n = 20)**	**Cycling (n = 20)**
Age (years)	56.8 ± 14.0	51.4 ± 18.1	-	-	-	-
Male Gender, n	12	12	-	-	-	-
Height (m)	1.67 ± 0.08	1.66 ± 0.11	-	-	-	-
Body Mass (kg)	80.49 ± 26.47	77.61 ± 18.27	79.96 ± 25.18	76.85 ± 17.87	81.48 ± 27.21	77.94 ± 18.80
BMI (kg/m^2)	28.67 ± 7.53	28.12 ± 6.44	25.80 ± 7.07	27.85 ± 6.29	28.97 ± 7.55	28.22 ± 6.52
Systolic BP (mmHg)	145 ± 19	142 ± 20	140 ± 21	140 ± 20	142 ± 22	142 ± 21
Diastolic BP (mmHg)	72 ± 11	74 ± 13	72 ± 12	73 ± 12	74 ± 11	73 ± 13
Ethnicity n (%)						
Caucasian	9 (45.0)	11 (55.0)	-	-	-	-
Asian	10 (50.0)	8 (40.0)	-	-	-	-
Black	1 (5.0)	1 (5.0)	-	-	-	-
Primary diagnosis n (%)						
Diabetic Nephropathy	7 (35)	2 (10)	-	-	-	-
Hypertensive Nephropathy	2 (10)	1 (5)	-	-	-	-
Glomerulonephritis	2 (10)	5 (25)	-	-	-	-
Pyelonephritis	1 (5)	1 (5)	-	-	-	-
Polycystic Kidney Disease	2 (10)	1 (5)	-	-	-	-
Other	1 (5)	4 (20)	-	-	-	-
Unknown	5 (25)	6 (30)	-	-	-	-
Previous Transplant, n (%)	2 (10)	3 (15)	-	-	-	-
Dialysis Vintage (months)	21.6 ± 19.3	33.0 ± 27.1	-	-	-	-
Comorbidities n (%)						
Hypertension	16 (80)	15 (75)	-	-	-	-
Coronary Artery Disease	3 (15)	5 (25)	-	-	-	-
Diabetes	9 (45)	5 (25)	-	-	-	-
Dyslipidaemia	4 (20)	7 (35)	-	-	-	-
Previous/Current Smoker	4 (20)	7 (35)	-	-	-	-
Medications, n (%)						
No. of Antihypertensives/day	1.7 ± 1.5	1.2 ± 1.0	-	-	1.6 ± 1.0	1.0 ± 0.9
No. of Phosphate Binders/day	2.6 ± 2.3	2.4 ± 2.7	-	-	2.4 ± 2.4	1.9 ± 2.0
Eprex dose/week (IU)	7350 ± 5040	7200 ± 7488	-	-	7620 ± 5602	6300 ± 7505
Iron dose/fortnight (IU)	80 ± 182	40 ± 50	-	-	115 ± 213	45 ± 51

Clinical Laboratory						
HbA1C (%)	6.30 ± 1.85	5.57 ± 1.24	6.80 ± 2.89	5.39 ± 1.16	5.78 ± 2.20	5.22 ± 0.74
Haemoglobin (g/L)	126.0 ± 14.4*	110.8 ± 28.9*	111.2 ± 12.7	119.0 ± 12.2	120.2 ± 14.1	116.0 ± 12.1
Ferritin (ig/L)	278 ± 150	288 ± 160	239 ± 133	310 ± 212	265.7 ± 140.2	261.5 ± 117.5
White Cell Count ($x10^9$/L)	7.86 ± 2.54	6.84 ± 2.16	7.03 ± 1.97	6.64 ± 1.57	7.67 ± 2.50	6.88 ± 1.96
Neutrophils ($x10^9$/L)	4.43 ± 1.37	4.22 ± 1.71	4.58 ± 1.18	4.39 ± 1.48	4.99 ± 1.51	4.12 ± 1.04
Lymphocytes ($x10^9$/L)	1.8 ± 0.5	1.7 ± 0.8	1.68 ± 0.60	1.48 ± 0.69	1.61 ± 0.60	1.73 ± 0.61
Monocytes ($x10^9$/L)	0.5 ± 0.2	0.5 ± 0.2	0.54 ± 0.09	0.48 ± 0.15	0.51 ± 0.17	0.45 ± 0.15
Platelets ($x10^9$/L)	216 ± 93	251 ± 213	225 ± 83	421 ± 698	214 ± 90	199 ± 59
Albumin (g/L)	33.8 ± 6.2*	38.1 ± 4.0*	34.3 ± 4.1	38.9 ± 4.2	35.1 ± 5.0	39.1 ± 5.34
Bicarbonate (mmol/L)	25.8 ± 2.8	34.6 ± 40.6	25.0 ± 2.0	26.6 ± 3.0	25.2 ± 2.5	27.7 ± 3.05
C-Reactive Protein (mg/L)	14.2 ± 23.0	19.3 ± 36.7	13.7 ± 16.9	11.9 ± 8.8	15.2 ± 34.6	17.1 ± 37.3
Parathyroid Hormone (pg/mL)	42.9 ± 43.3	54.0 ± 51.5	52.6 ± 60.1	73.6 ± 63.3	65.0 ± 54.1	81.4 ± 63.5
Calcium (mmol/L)	2.40 ± 0.21	2.33 ± 0.16	2.43 ± 0.16	2.32 ± 0.20	2.33 ± 0.14	2.27 ± 0.18
Phosphate (mmol/L)	1.48 ± 0.38*	1.83 ± 0.66*	1.58 ± 0.60	1.85 ± 0.49	1.59 ± 0.37	1.84 ± 0.71
Total Cholesterol (mmol/L)	3.93 ± 0.98	4.29 ± 1.25	4.08 ± 1.08	4.29 ± 1.70	3.98 ± 1.17	4.39 ± 1.49
Triglycerides (mmol/L)	1.85 ± 1.50	1.65 ± 1.02	1.90 ± 1.24	1.76 ± 1.28	1.64 ± 0.98	1.74 ± 0.71
Urea Reduction Ratio (%)	74 ± 14	77 ± 8	78 ± 6	75 ± 13	76 ± 6	75 ± 9

Table 5.1. Demographic and clinical data. Medication data was only collected at baseline and final timepoints. No significant impact of the intervention was recorded in any variable * = significant baseline difference between groups (p = .047, .014 and .047, respectively). Data are presented as 'mean ± SD'. All data presented in this table was normally distributed. (Control n = 20, IDC n = 20).

vascular access (4%); patient was significantly fluid-overloaded (2%); patient reported fatigue (1%) and other miscellaneous reasons (1%).

The mean session duration, cadence and RPE remained unchanged throughout the intervention ($p \geq .593$, $\eta^2 \leq .038$). However, the mean gear achieved during each session displayed a significant effect of time ($p < .001$, $\eta^2 = .288$). Post-hoc analysis revealed that the patients achieved a significantly higher gear in months 5 and 6 compared to month 1 ($p = .026$ and $p = .024$, respectively). However, despite this progression in gear and encouragement from the exercise supervisors to increase session intensity, the mean estimated distance, power and estimated energy expenditure achieved during each session remained unchanged throughout the intervention ($p \geq .368$, $\eta^2 \leq .055$) ($n = 46$ required to detect significant changes). The monthly exercise session variables are presented in Figures 5.3 and 5.4.

Physical function

The control group had a significantly higher STS5 time-to-completion than the exercise group at baseline ($p = .015$). However, the groups were well-matched at baseline for STS60 score and ISWT duration, post-ISWT RPE and post-ISWT heart rate ($p \geq .115$). The exercise intervention had no effect on the time taken to complete the STS5. There was no time*group interaction effect ($p = .417$, $\eta^2 = .033$). Similarly, the number of repetitions achieved in the STS60 showed no time*group interaction effect ($p = .382$, $\eta^2 = .032$). Finally, the distance achieved in the ISWT, the post-ISWT RPE and the post-ISWT HR showed no time*group interaction effect ($p \geq .406$, $\eta^2 \leq .028$). This data is presented in Table 5.2 and Figure 5.5. Post-hoc sample size calculations suggested that total sample sizes of $n = 78$, $n = 80$ and $n = 92$ would have been necessary to detect significant changes in the STS5 (time), STS60 (reps) and ISWT (distance), respectively in response to the exercise intervention (interaction effect).

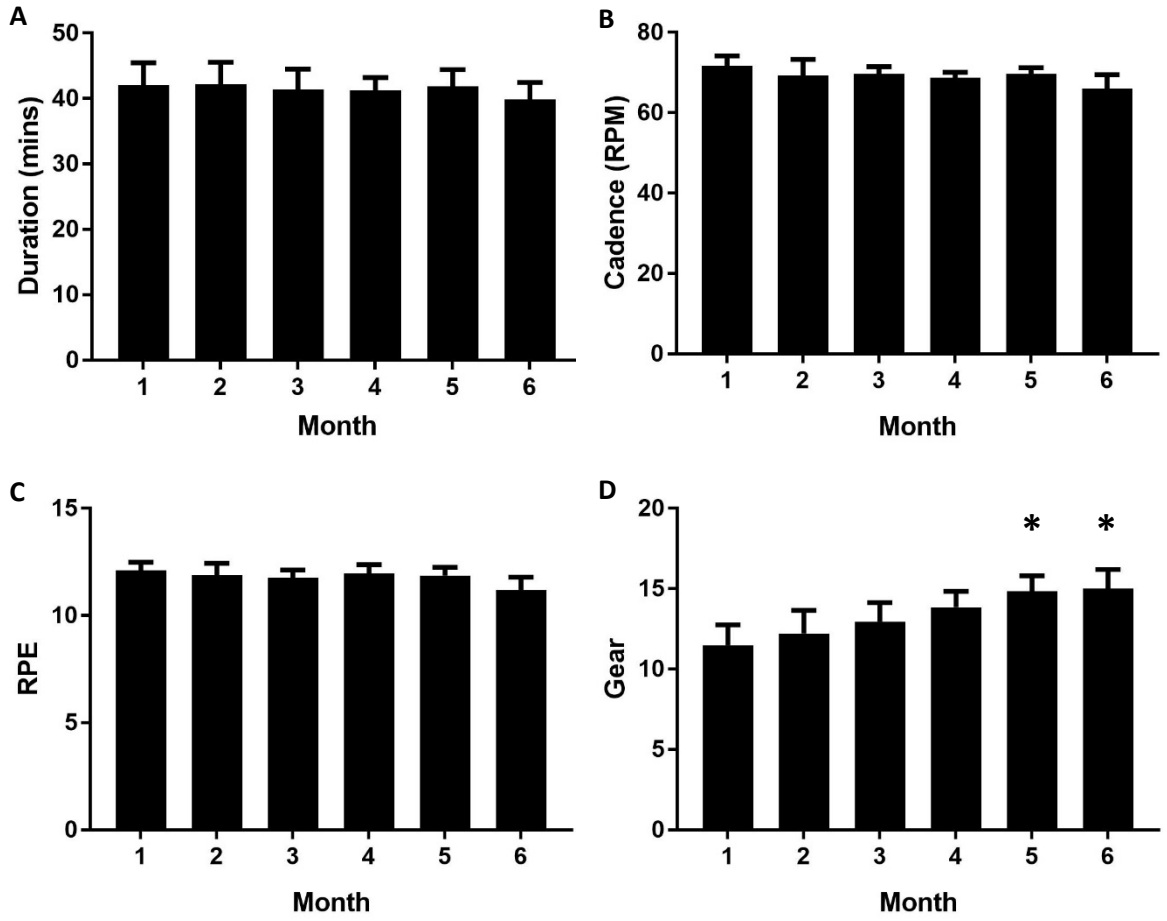

Figure 5.3. Month-by-month mean session duration (A), cadence (B), RPE (C) and gear (D). Data are presented as 'mean ± SEM'. * = significantly different from month 1 (p = .026 and p = .024, respectively). This data was normally distributed. (IDC only, n = 20).

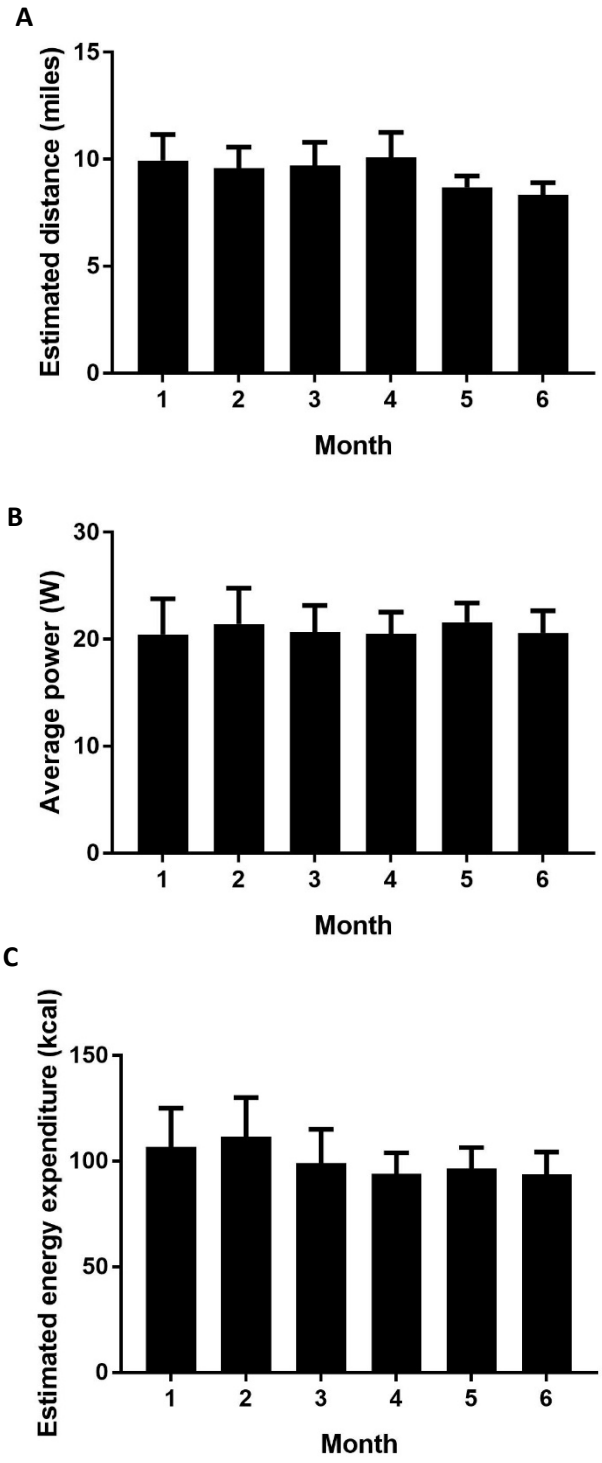

Figure 5.4. Month-by-month mean session estimated distance (A), average power (B) and estimated calorie expenditure (C). Data are presented as 'mean ± SEM'. This data was normally distributed. (IDC only, n = 20).

Timepoint	Baseline		Interim		Final	
Group	**Control (n = 20)**	**Cycling (n = 20)**	**Control (n = 20)**	**Cycling (n = 20)**	**Control (n = 20)**	**Cycling (n = 20)**
STS5 (s)	17.20 ± 6.95*	11.30 ± 6.36*	18.45 ± 6.36	11.30 ± 5.91	16.98 ± 5.75	10.12 ± 5.00
STS60 (reps)	17 ± 12	23 ± 10	17 ± 13	28 ± 16	17 ± 13	23 ± 11
IWST distance (m)	254 ± 156	314 ± 174	269 ± 154	295 ± 163	253 ± 166	314 ± 188
ISWT RPE	13 ± 2	12 ± 2	13 ± 2	13 ± 1	13 ± 2	13 ± 1
Post-ISWT HR (bpm)	92 ± 23	100 ± 20	94 ± 22	97 ± 23	90 ± 16	92 ± 24

Table 5.2. Physical function results throughout the intervention. * = significant baseline difference between groups (p = .015). No significant impact of the intervention was recorded in any variable. Data are presented as 'mean ± SD'. All data presented in this table was normally distributed. (Control n = 20, IDC n = 20).

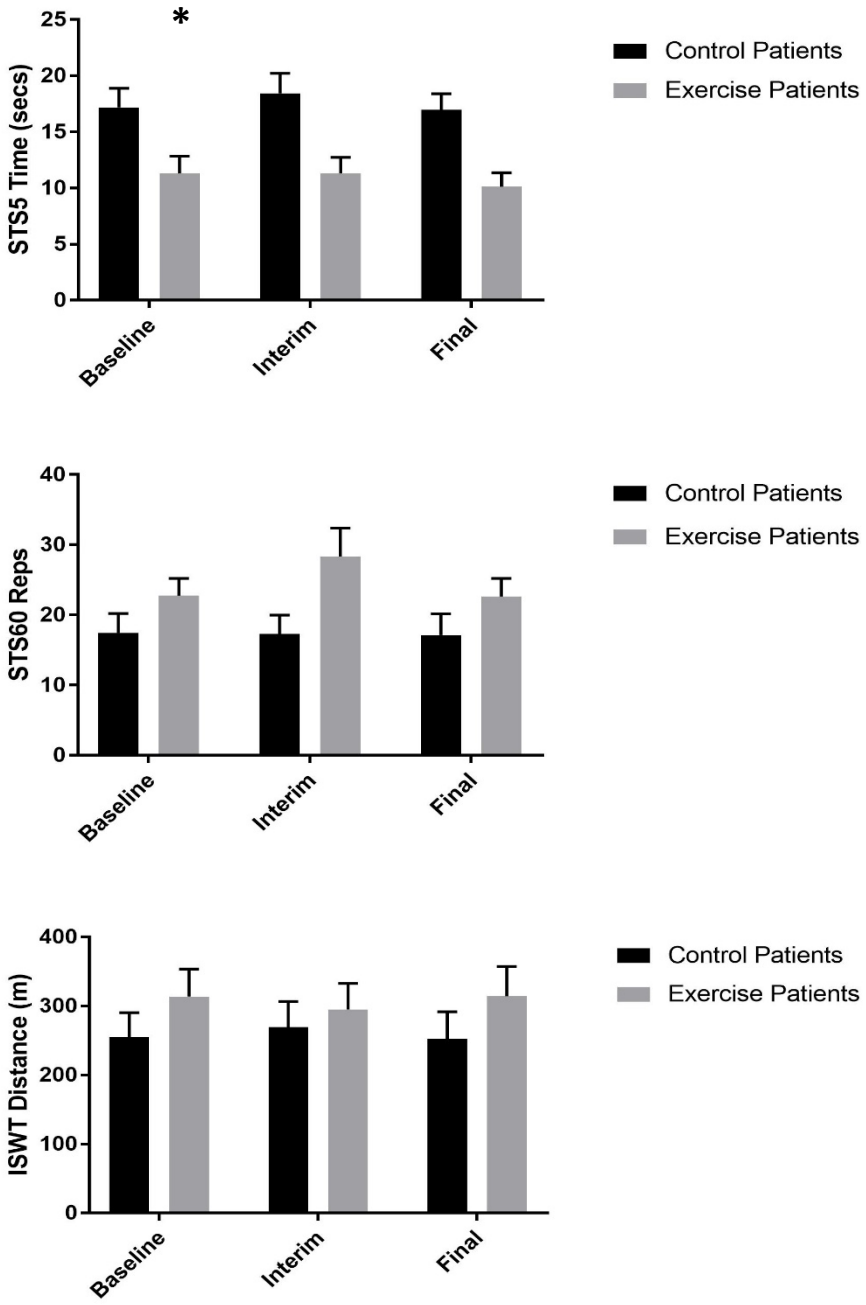

Figure 5.5. Exercise performance data at baseline, interim and final timepoints. * = significantly lower than control group (p = .015). Data are presented as 'mean ± SEM'. This data was normally distributed. (Control n = 20, IDC n = 20).

Circulating cytokines

All assessed circulating IL-2 concentrations in this study fell below the minimum detectable threshold and as such were indistinguishable from the background noise inherent in the technique. As such, no IL-2 data is presented as the technique was not able to produce valid values for this cytokine.

Conversely, all samples (n = 40) provided valid and detectable IL-6 values. Circulating IL-6 levels showed no differences at baseline (p = .094) and no significant time*group interaction effect (p = .154, η^2 = .056) (n = 46 necessary to detect significant interaction effect). This is displayed in Figure 5.6.

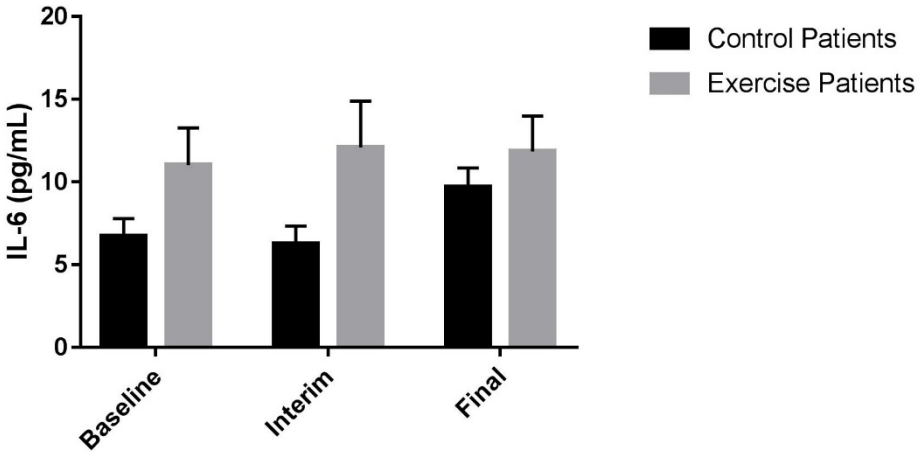

Figure 5.6. Circulating IL-6 levels. Data are presented as 'mean ± SEM'. This data was non-normally distributed and thus logarithmically transformed prior to analysis. (Control n = 20, IDC n = 20).

As with IL-6, all IL-10 values fell above the minimum detectable threshold. No significant baseline group differences were observed (p = .466) and no time*group interaction effect was observed (p = .060, η^2 = .076). This is displayed in Figure 5.7. Given the effect size presented, post-hoc sample size calculations suggested that a sample size of n = 34 would be necessary to detect an interaction effect –as such it is likely that that elevated variations prevented a significant finding from being detected here.

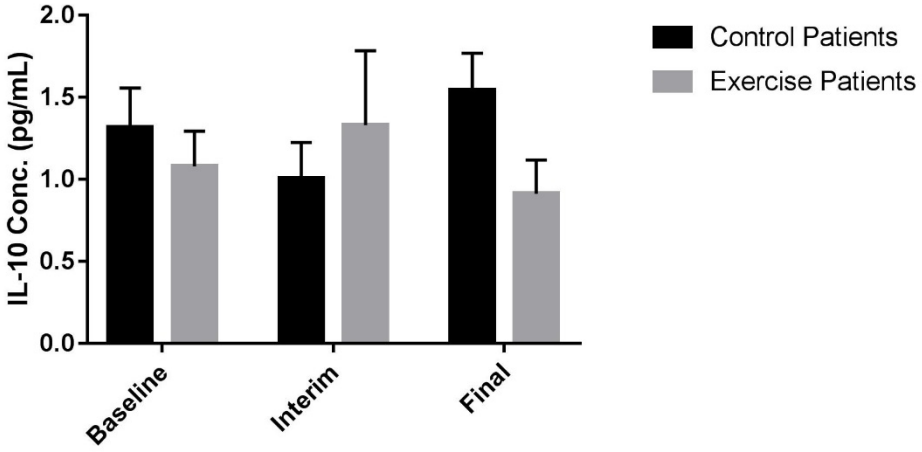

Figure 5.7. Circulating IL-10 levels. Data are presented as 'mean ± SEM'. This data was logarithmically transformed prior to statistical analysis. (Control n = 20, IDC n = 20).

Similarly to IL-2, the majority of IL-17a findings from the samples assessed here fell below the minimum detectable threshold of 0.274 pg/mL. Only two of the control patients and seven of the IDC patients displayed valid results. Whilst these values underwent the same statistical analysis as the other included cytokines, the results should be interpreted with caution given their low sample size. No baseline group differences were observed ($p = .262$) and no time*group interaction effect was observed ($p = .321$, $\eta^2 = .150$). This data was non-normally distributed and thus was logarithmically transformed prior to analysis. Post-hoc calculations suggested that a sample size of n = 18 would be necessary to detect significant alterations in IL-2 levels (n = 9 used here due to the removal of invalid data).

Regarding TNF-α, valid results above the minimum detectable threshold were obtained for nine of the control patients and 12 of the IDC patients. No group differences were observed at baseline ($p = .500$), however there was a significant time*group interaction effect ($p = .001$, $\eta^2 = .322$). Further post-hoc testing revealed that there was a significant reduction in the IDC condition from baseline to final ($p < .001$, $\eta^2 = .272$), that was not observed in the control group ($p = .834$, $\eta^2 = .022$). This is presented in Figure 5.8.

Lastly, the ratio of circulating IL-6 to IL-10 levels displayed significant group differences at baseline ($p = .041$, $\eta^2 = .144$). However, no time*group interaction effect was revealed ($p = .860$, $\eta^2 = .001$). This is displayed in Figure 5.9.

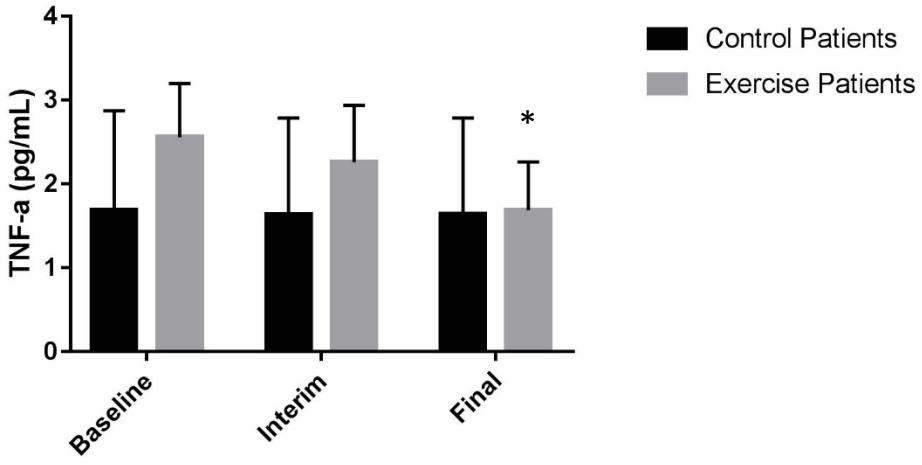

Figure 5.8. Circulating TNF-α. Data are presented as 'mean ± SEM'. * = significantly different from baseline. This data was logarithmically transformed prior to statistical analysis. (Control n = 9, IDC n = 12).

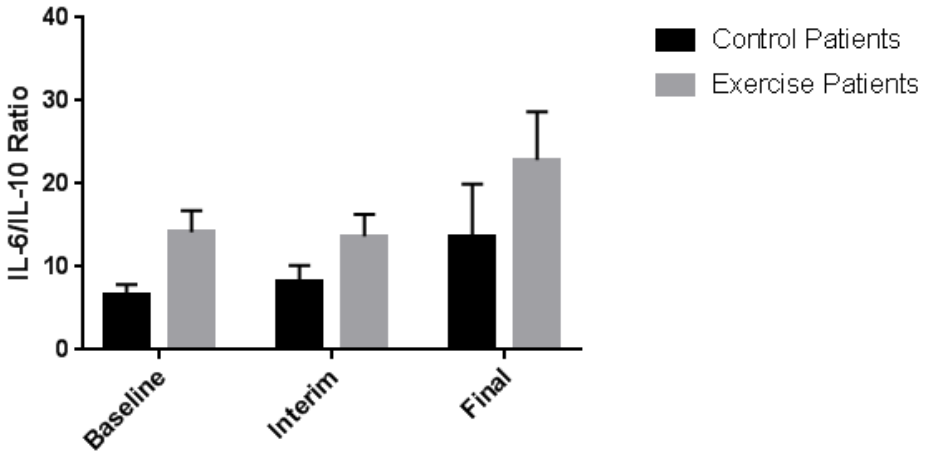

Figure 5.9. IL-6/IL-10 ratio. Data are presented as 'mean ± SEM'. This data was logarithmically transformed prior to statistical analysis. (Control n = 20, IDC n = 20).

Circulating chemokines

All chemokine data was normally distributed. Circulating chemokine concentrations (IL-8, MIG, MCP-1 and IP-10) all displayed no significant baseline differences ($p \geq .101$) and showed no time*group interaction effects ($p \geq .151$, $\eta^2 \leq .053$). Post-hoc sample size analysis suggested that a sample size of at

least n = 48 would be necessary to elicit detectable significant alterations in chemokine levels (interaction effect). All chemokine results are presented in Table 5.3.

	Control (n = 20)			Cycling (n = 20)		
Chemokine	**Baseline**	**Interim**	**Final**	**Baseline**	**Interim**	**Final**
IL-8 (pg/mL)	23.70 ± 15.60	20.09 ± 13.90	21.71 ± 17.80	20.68 ± 9.61	20.00 ± 10.51	21.41 ± 11.67
MIG (pg/mL)	436.27 ± 349.15	412.39 ± 324.16	441.52 ± 359.50	662.67 ± 537.09	544.38 ± 862.25	530.42 ± 470.14
MCP-1 (pg/mL)	47.06 ± 23.47	35.30 ± 10.20	44.64 ± 18.25	39.81 ± 25.31	39.65 ± 22.94	41.30 ± 24.55
IP-10 (pg/mL)	220.37 ± 100.79	219.70 ± 119.04	250.76 ± 159.56	298.80 ± 182.55	243.04 ± 136.13	260.17 ± 160.72

Table 5.3. All circulating chemokine data. Percent (%) change values were calculated from baseline to interim, and from baseline to final timepoints. Data is presented as 'mean ± SD'. This data was normally distributed and thus not logarithmically transformed prior to analysis. (control n = 20, IDC n = 20).

Circulating microparticles

Both total MPs (based on scatter profile only) and Annexin-V$^+$ MPs (used as a marker of phosphatidylserine expression) showed no baseline group differences ($p \geq .326$) and no time*group interaction effects ($p \geq .725$, $\eta^2 \leq .009$).

Similarly, the circulating concentration of total platelet-derived MPs, the circulating concentration of TF$^+$ platelet-derived MPs, the % of total MPs that were platelet-derived MPs and the % of platelet-derived MPs that were TF$^+$ all showed no baseline differences ($p \geq .279$) and no significant time*group interaction effects ($p \geq .304$, $\eta^2 \leq .035$) (required sample size of at least n = 74 to elicit detectable significant differences in these variables).

Whilst the number of neutrophil-derived MPs displayed no baseline group differences ($p = .279$), a trend towards a significant time*group interaction effect was revealed ($p = .051$, $\eta^2 = .096$). Further analysis revealed that whilst no differences were seen between groups at baseline and interim ($p \geq .708$), there was an increase in the control group only from interim to final that created a trend towards significance between groups at this timepoint ($p = .063$). This relationship is displayed in Figure 5.10. However, no other neutrophil-derived MP variable (TF$^+$ neutrophil-derived MPs, % of total MPs that were neutrophil-derived MPs, % of neutrophil derived MPs that were TF$^+$) displayed significant time*group interaction effects ($p \geq .261$, $\eta^2 \leq .040$) (at least n = 64 required to elicit significant detectable changes in these variables from post-hoc calculation).

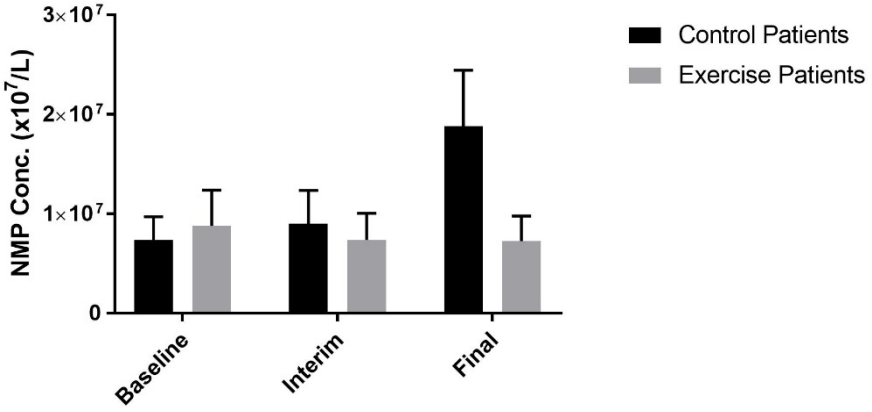

Figure 5.10. Circulating concentration of neutrophil-derived MPs (NMP). Data are presented as 'mean ± SEM'. This data was logarithmically transformed prior to analysis. (Control n = 20, IDC n = 20).

Similar to the platelet-derived MPs, the circulating concentration of monocyte-derived MPs, the circulating concentration of TF^+ monocyte-derived MPs, the % of total MPs that were monocyte-derived MPs and the % of monocyte-derived MPs that were TF^+ all showed no baseline differences ($p \geq .343$) or significant time*group interaction effects ($p \geq .456$, $\eta^2 \leq .035$) (at least n = 74 required).

The number of endothelial cell-derived MPs displayed no significant time*group interaction effect ($p = .097$, $\eta^2 = .068$) (Figure 5.11). Additionally, the number of TF^+ endothelial cell-derived MPs, the % of total MPs that were endothelial cell-derived and the % of endothelial cell-derived MPs that were TF^+ (Figure 5.12) all displayed no time*group interaction effect ($p \geq .217$, $\eta^2 \geq .399$).

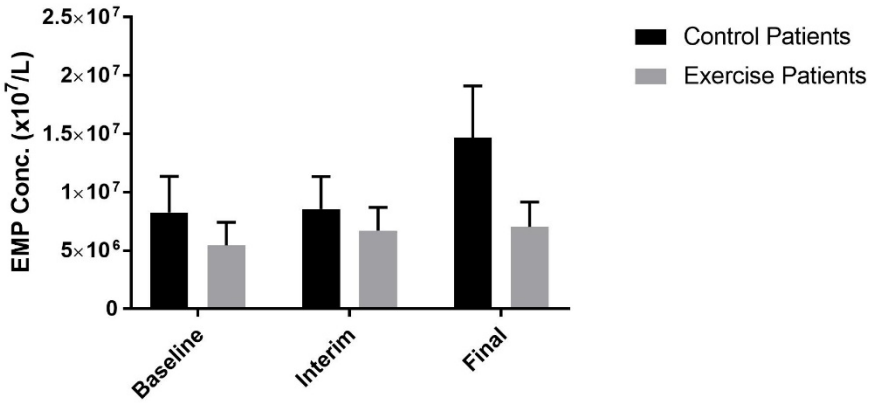

Figure 5.11. Circulating concentration of endothelial cell-derived MPs. Data are presented as 'mean ± SEM'. This data was logarithmically transformed prior to analysis. (Control n = 20, IDC n = 20).

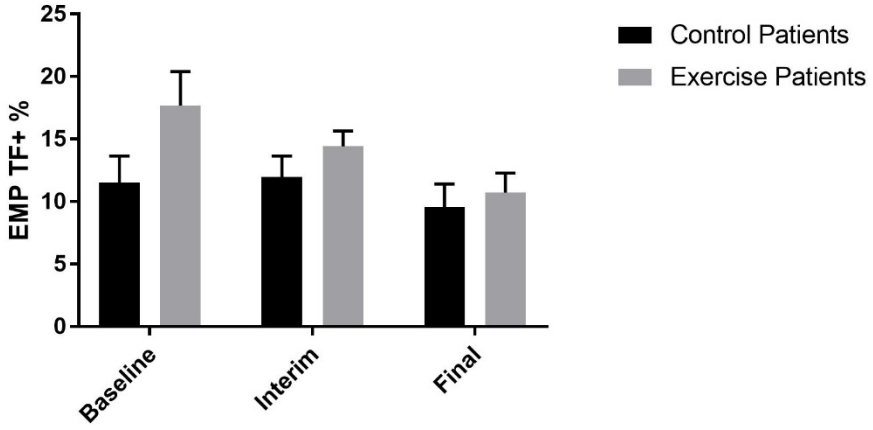

Figure 5.12. Circulating percentage of TF-positive endothelial cell-derived MPs (of total EMP pool). Data are presented as 'mean ± SEM'. This data was normally distributed and thus was not transformed prior to analysis. (Control n = 20, IDC n = 20).

The remaining MP phenotype and TF expression data that is not displayed graphically is presented in Table 5.4.

	Control (n = 20)			Cycling (n = 20)		
	Baseline	**Interim**	**Final**	**Baseline**	**Interim**	**Final**
***Total MP conc.** ($x10^9$/L)	12.30 ± 10.96	12.26 ± 18.29	17.96 ± 23.57	14.76 ± 17.84	22.29 ± 29.01	20.71 ± 20.08
***AnV$^+$ MP conc.** ($x10^7$/L)	13.59 ± 9.30	9.08 ± 7.02	17.03 ± 12.25	11.15 ± 7.20	10.56 ± 7.20	12.72 ± 6.98
***PMP conc.** ($x10^7$/L)	7.91 ± 4.52	6.33 ± 7.56	8.48 ± 5.63	9.19 ± 3.98	10.21 ± 7.87	8.87 ± 6.08
TF$^+$ PMP conc. ($x10^7$/L)	2.26 ± 1.43	1.72 ± 1.61	1.98 ± 1.57	2.69 ± 1.65	2.52 ± 1.30	2.41 ± 1.52
% PMP of total MPs	1.59 ± 1.92	0.85 ± 1.03	1.75 ± 2.59	1.99 ± 2.73	1.42 ± 1.61	1.84 ± 2.70
% PMP TF$^+$	28.46 ± 14.40	29.60 ± 15.52	23.70 ± 12.25	29.83 ± 13.91	32.30 ± 12.79	31.08 ± 11.98
***TF$^+$ NMP conc.** ($x10^7$/L)	0.13 ± 0.17	0.24 ± 0.40	0.20 ± 0.31	0.17 ± 0.27	0.11 ± 0.18	0.15 ± 0.22
% NMP of total MPs	0.24 ± 0.49	0.08 ± 0.13	0.20 ± 0.36	0.21 ± 0.54	0.07 ± 0.13	0.11 ± 0.27
% NMP TF$^+$	19.84 ± 12.25	23.57 ± 11.05	18.90 ± 8.77	23.78 ± 10.69	26.21 ± 10.29	23.60 ± 5.99
***MMP conc.** ($x10^7$/L)	3.82 ± 3.04	3.78 ± 3.53	3.91 ± 3.35	5.03 ± 3.53	4.21 ± 3.49	4.00 ± 3.94
***TF$^+$ MMP conc.** ($x10^7$/L)	0.84 ± 0.76	0.73 ± 0.80	0.86 ± 0.89	1.24 ± 0.98	0.85 ± 0.67	0.97 ± 1.34
% MMP of total MPs	0.81 ± 1.57	0.51 ± 0.80	1.14 ± 2.15	0.97 ± 1.48	0.72 ± 0.81	1.09 ± 1.92
% MMP TF$^+$	21.16 ± 7.60	18.92 ± 6.75	20.56 ± 9.97	24.14 ± 10.00	20.92 ± 8.54	23.90 ± 6.39
***TF$^+$ EMP conc.** ($x10^7$/L)	0.06 ± 0.13	0.09 ± 0.13	0.05 ± 0.09	0.06 ± 0.09	0.10 ± 0.13	0.08 ± 0.09
% EMP of total MPs	0.10 ± 0.18	0.16 ± 0.31	0.19 ± 0.45	0.08 ± 0.18	0.26 ± 0.40	0.32 ± 0.63

Table 5.4. MP phenotype and TF expression data. AnV = Annexin-V; PMP = platelet-derived MP; NMP = neutrophil-derived MP; MMP = monocyte-derived MP; EMP = endothelial cell-derived MP; TF = tissue factor. All data is presented as 'mean ± SD'. * indicates that data was non-normally distributed and thus was logarithmically transformed prior to analysis. (Control n = 20, IDC n = 20).

5.5 Discussion

The aim of this study was to investigate the effects of 6-months of regular, moderate intensity IDC on markers of inflammation in unit-based HD patients. The exercise intervention had no effect on the demographic and clinical results or the physical function test results. However, IDC reduced the circulating concentration of the circulating pro-inflammatory cytokine TNF-α, whilst circulating chemokines and microparticles remained largely unchanged.

Demographics and clinical information

Groups were well matched at baseline for all demographic and anthropometric measures, and well matched for comorbidity prevalence, primary diagnoses, medications and the majority of the clinical laboratory results. The significant differences shown in haemoglobin, albumin and phosphate levels at baseline between groups may be partially down to the heterogeneity of the haemodialysis population, but also due to the relatively small sample size and also potentially the influence of the cohort selection procedure. However, the cohort selection procedure was largely successful in producing two matched groups at baseline.

Exercise compliance and progression

The exercise compliance of the intervention was relatively high (82%). This highlights one of the benefits of IDC training programmes, in that patient compliance is high as the training is completed during time they are already sacrificing for dialysis treatment. Additionally the month-by-month mean session characteristics suggest that the exercise sessions were progressed appropriately. Mean session duration, RPE and cadence remained constant, whilst the average gear achieved was progressively increased, thus representing the traditional pattern of progressive overload and adaptation. However, this progressive overload protocol did not result in significant increases in the mean estimated distance, estimated energy expenditure or power of each session. Whilst distance and energy expenditure are only estimated values (as the true values would be influenced by patient body mass) it is unusual that an increase was not seen. Similarly, as mean cadence remained constant and the mean gear achieved was gradually increased, it is also unexpected that mean session power did not increase. Combined, this

lack of changes would suggest an absence of adaptation to training, despite the patients being able to tolerate an increasingly higher cycling resistance throughout the intervention without an increase in RPE. A possibility is that once the novelty of the opportunity to regular participate in IDC training had worn off, motivation levels may have diminished and thus prevented further exercise progression. However, exercise motivation levels were not monitored in this study and thus this remains only speculative.

Physical function

Given the lack of significant changes seen in the exercise session characteristics, it is perhaps unsurprising that the physical function test results were unresponsive to the exercise intervention. Previous research regarding the effects of longitudinal IDC training on markers of physical performance and function has provided mixed results – some studies have found significant improvements in physical function tests (e.g. six-minute walk distance) (Parsons et al. 2006), whilst other studies have found no influence of 6-months of regular IDC training on six-minute walk distance (Koh et al. 2010). A recent systematic review and meta-analysis found that whilst IDC training does not improve VO_2 peak, significant increases in six-minute walk distance have been demonstrated when considering the whole body of literature (Young et al. 2018). The six-minute walk test was not included in this study, however previous research has demonstrated that six months of regular IDC training can elicit significant improvements in sit-to-stand 60 performance (Dungey et al. 2017), a surrogate measure of muscular power (Lindemann et al. 2003), which was included here but demonstrated no significant improvement. When regarding the sit-to-stand 60 results (Figure 5.5), there was a slight return to baseline from the interim to final measures, resulting in no overall significant increase, which would lend credence to the concept of diminished motivation following familiarity with the intervention as mentioned earlier. It is also possible that, given the functional testing completed was of a different exercise modality that the training completed (cycling vs walking/sit-to-stand), this may have prevented improvements being observed. However, more likely is that the low exercise intensity was not sufficient enough to induce exercise adaptation. The post-hoc sample size calculations suggested that a sample size of at least n =

78 would be necessary to elicit statistically significant improvements in the exercise group given the effect sizes presented earlier.

Circulating cytokines

The number of invalid findings in IL-2 and IL-17a point towards their low circulating concentrations in HD patients. This suggest that they may not be as clinically relevant as the more traditionally measured IL-6, IL-10 and TNF-α, which all showed largely valid results (TNF-α was detected in ~50% of the samples). The reduction in TNF-α is an encouraging finding, as TNF-α has been associated with elevated skeletal muscle protein breakdown rates, increased presence of carotid plaques, calcification of vascular cells and endothelial dysfunction in ESRD patients (Stenvinkel et al. 2005).

Previous research concerning IDC training and markers of systemic inflammation are limited and have provided mixed results; some have shown an overall reduction in circulating IL-6 and CRP after 3 months (Liao et al. 2016), whilst others have shown no change in IL-6, TNF-α or CRP after 6 months (Dungey et al. 2017). Interestingly, despite showing no changes in markers of inflammation, Dungey et al found that 6 months of regular IDC induced a favourable shift in circulating intermediate monocyte proportion (reduction) and regulatory T cell number (relative increase compared to control). It is unclear why this did not result in alterations in circulating cytokine concentrations, especially given that intermediate monocytes secrete the greatest amount of IL-6 when compared to the classical and non-classical subsets (Rossol et al. 2012). However, in this study, only total monocyte and lymphocyte numbers were assessed and so it is not known if any phenotypic shifts occurred within each leukocyte type. Lastly, it could be proposed that the lack of change in circulating CRP (a positive acute phase reactant) is unusual given the fluctuations in circulating cytokine levels. However, CRP is more closely linked with IL-6 (which remained unchanged) than TNF-α (Stenvinkel et al. 2005) and as such was also unaffected. In summary, whilst IL-6 and IL-10 levels were unaffected by the intervention, the TNF-α results displayed a potentially anti-inflammatory effect. However, these findings may be slightly blurred by the lack of valid results seen in some cases – this may warrant further investigation with larger

sample sizes to clarify the impact of samples displaying TNF-α values that are below the detectable limit.

Circulating chemokines

Chemokines are a family of cytokines that attract and induce chemotaxis of specific cells (Graves and Jiang 1995). In the case of the chemokines assessed here, they are primarily secreted by cells resident within the vascular wall (usually the intima or media) and therefore, in combination with adhesion molecules such as VCAM-1, attract leukocytes to the vascular endothelium and promote cellular adhesion and extravasation. This is a necessary response to injury or infection, however when unregulated, chronically elevated chemokine secretion can promote elevated cellular extravasation and ultimately the propagation of atherosclerotic plaque. From the little research concerning circulating chemokine concentrations in ESRD and HD patient populations, it has been suggested that certain chemokines, such as MCP-1, are chronically elevated and that these elevations are positively associated with carotid atherosclerosis (as measured by intima-media thickness) (Papayianni et al. 2002; Papayianni et al. 2003). However, to the author's knowledge, there is no research concerning exercise in these populations. In patients with metabolic syndrome, 12 weeks of regular mixed aerobic and resistance training resulted in reduced circulating concentrations of MCP-1 and IL-8 (Trøseid et al. 2004), the chemokines specific for monocytes and neutrophils, respectively. A possible explanation for this effect is a reduction in tissue infiltration by leukocytes (primarily macrophages) resulting in a reduction in the number of tissue-resident cells able to secrete chemokines. Chemokines perhaps represent an under-researched branch of the cytokine family. Therefore, further research should include measures of cytokines, chemokines and adhesion molecules in order to capture the whole story of how inflammation leads to endothelial dysfunction, atherosclerosis and vascular disease. This would better provide a 'start-to-finish' image of the pathophysiology involved and may help to increase understanding of the pathways linking regular aerobic exercise to improved vascular health and decelerated cardiovascular disease development.

Circulating microparticles

Firstly, although not reaching statistical significance (time*group p = .051), there seemed to be a progressive increase in the number of neutrophil-derived MPs in the control condition which was not observed in the exercise condition, creating a difference between groups at this timepoint that approached significance (p = .063). This suggests that regular IDC can prevent an increase in the circulating concentration of neutrophil-derived MPs. The number of possible causes of this effect are numerous, but most likely include: an effect of regular increased blood flow due to aerobic exercise; a reduction in neutrophil activation caused by the reduction in pro-inflammatory mediators presented earlier; a reduction in neutrophil activation in response to a reduction in circulating uraemic toxins or retention solutes; or a reduction in the rates of apoptosis of neutrophils. Previous research has shown that 5 weeks of regular (30 min, 5x/wk), moderate intensity (60% VO_2 max) cycling training can reduce the circulating concentration of neutrophil-derived MPs, and prevent the increase seen in neutrophil-derived MP formation in response to acute hypoxic exercise (Chen et al. 2015). As HD patients commonly experience intradialytic hypotension (Palmer and Henrich 2008) and reduced blood flow kinetics (McIntyre et al. 2008), it may be that regular IDC training restores systemic circulation and prevents a hypoxic environment, thus preventing an increase in neutrophil-derived MP formation. Murine research has also shown that administration of TNF-α increases the rates of neutrophil-derived MP formation (Johnson et al. 2013). Therefore, given circulating TNF-α levels seemed to decline in the exercise condition only, this may have prevented an increase in MP formation.

Uraemic toxins can act as ligands for leukocytes and thus cause cellular activation (Pletinck et al. 2013), resulting in increased MP formation (Meijers et al. 2009). Regular IDC has been proposed as a method to increase uraemic toxin removal during dialysis by preventing blood accumulation in the lower limbs and increasing the amount of blood available for filtration (Kong et al. 1999; Orcy et al. 2014; Parsons et al. 2006). Whilst no changes were seen in uraemic toxin concentrations in this study, only three were measured (phosphate, urea, creatinine) and many other uraemic toxins have been reported in previous literature (e.g. p-cresol, indoxyl sulphate) (Duranton et al. 2012). As such, a training-induced reduction

in circulating uraemic toxin concentration may have been responsible for the prevention of increased neutrophil-derived MP formation. However, it must be noted that not all studies demonstrate increased uraemic toxin removal in response to intradialytic exercise training (Böhm et al. 2017). Lastly, whilst the HD procedure has been shown to elicit increased rates of leukocyte apoptosis (Majewska et al. 2003; Moser et al. 2003), there is little evidence to support the notion that intradialytic exercise can ameliorate this effect. It must also be considered that, rather than a reduction in the circulating concentration of neutrophil-derived MPs, there could have been an alteration in the protein expression of these MPs thus falsely representing a reduction. As only one marker was used to distinguish these MPs (CD66b) this theory cannot be supported or discredited here.

As explained in Chapter 2, increased shear stress elicited by increased blood flow can increase endothelial cell-MP formation. However, the actual nature of this stimulus seems to be a departure from membrane quiescence. Given the reduction in vascular shear stress observed in HD patients (as a result of intradialytic hypotension and an anaemia-induced reduction of blood viscosity) (Boulanger et al. 2007), a restoration of adequate blood flow during HD may actually increase shear stress to 'normal levels' and restore membrane quiescence, thus reducing the shedding of endothelial cell-derived MPs. This effect has been reported previously by manipulating shear stress on cultured human endothelial cells and has been reported to be mediated by increased mitochondrial biogenesis and maintenance of mitochondrial integrity within endothelial cells (Kim et al. 2015). However, this effect was not observed here as no significant effects were observed with regards to endothelial microparticles, perhaps again due to the relatively low mean session exercise intensity.

Regardless of the cause, a reduction in circulating neutrophil-derived MPs would be expected to elicit a positive response with regards to systemic inflammation, thrombosis and cardiovascular risk, though it must be noted again that this interaction effect did not reach statistical significance ($p = .051$). Further research should include more markers for phenotype-specific MPs in order to better characterise their response to aerobic exercise training regimes.

Limitations

This study design includes several limitations which must be acknowledged. Firstly, it seems that the mean exercise intensity achieved by the HD patients was low (~20 W). This may have limited the adaptation to training, resulting in the general lack of significant changes seen in response to the intervention. Similarly, whilst the exercise intervention employed cycling as an exercise modality, the physical function testing consisted of walking and sit to stand tests. As adaptations to exercise training are highly modality-specific (Hawley 2008), this may have limited the sensitivity of the physical function tests to possible exercise adaptations. However, the STS and ISWT tests were considered to be easy and simple tests and as such were used instead of cycle ergometer VO_2peak tests in order to minimise patient and investigator burden. Additionally, whilst the whole study was randomised and adequately powered using a peer-reviewed protocol, this study incorporated only a cohort of the entire study. As such, reducing the included sample size may have limited statistical power, and the cohort selection procedure may have unintentionally introduced bias. This would also explain the differences seen at baseline in some of the measured inflammatory mediators. The coefficient of variations of the cytometric bead array and MP analysis techniques (10.08% and 13.88%, respectively) are large and therefore increase the chance of Type 2 error. Also, the cytometric bead array technique was unable to detect circulating IL-2, meaning this cytokine cannot be discussed regarding the results obtained here. It was also largely unable to detect circulating IL-17a and only detected circulating TNF-α in roughly 50% of cases, limiting the relevance and validity of the observed findings.

Lastly, as is presented in Figure 5.1, there were a large number of patient dropouts or incompletes (n = 64/128 when selecting the cohort included here from the entire study cohort) that could not be included in the analyses presented here. Large numbers of dropouts can potentially insert a degree of bias and impact the validity of the findings of randomised controlled trials (Bell et al. 2013), as the remaining sample included in the analyses may not be truly representative of the population as a whole. The notion that the analysed sample may not be truly representative of the HD population is important as it suggests that the IDC intervention may not have been suitable for a certain proportion of HD

patients. Additionally, the results may represent a population that find IDC more suitable than the HD population as a whole and may skew the findings towards favouring IDC, which could be misleading as it does not actually truly represent the desired population. This is also important for the design of future studies investigating IDC in this population. If only a certain proportion of HD patients find IDC to be an acceptable form of exercise, the overall efficacy of this exercise modality may be limited when attempting to implement it into routine clinical practice in a non-research-based setting. Additionally, recent research has suggested that the number of clinically meaningful responders to IDC may be as low as < 50% (Valenzuela et al. 2018). Therefore, future studies may benefit from attempting to identify those patients that will both find the intervention tolerable and exhibit a clinically meaningful response. Larger randomised controlled trials will allow the stratification of patients based on relevant demographic and clinical variables (e.g. age, physical function, HD vintage, primary diagnosis, co-morbidities) in order to identify patients most likely to benefit and therefore create more targeted and individualised interventions in the future.

Conclusions and future research

In conclusion, a 6-month programme of thrice-weekly intradialytic cycling training, whilst not inducing significant improvements in physical performance, reduced the circulating concentration of circulating TNF-α and did not significantly impact circulating chemokines or MPs. Future research should aim to investigate this relationship in larger sample sizes with a view to stratifying patient groups based on factors such as HD vintage and primary diagnosis, as these may impact the inflammatory environment. Additionally, given the findings of the post-hoc sample size calculations here, studies looking to demonstrate improved physical function as a result of regular IDC training should employ much larger sample sizes.

Chapter 6

Study 3

The effects of intradialytic low frequency electrical muscle stimulation on markers of systemic inflammation in haemodialysis patients

6.1 Abstract

Haemodialysis (HD) patients display elevated cardiovascular risk and all-cause mortality when compared to the general population, which may be attributed to non-traditional risk factors such as inflammation, oxidative stress and uraemia. Intradialytic cycling (IDC) is a possible therapeutic strategy to combat chronically elevated systemic inflammation and thus reduce cardiovascular risk, however some patients with severe functional limitations may not be able to complete volitional exercise training. Low-frequency electrical muscle stimulation (LF-EMS) is a possible alternative for these patients, and preliminary evidence suggests it may improve physical conditioning and reduce markers of inflammation in HD patients (described in Chapter 2). This study investigated the effects of a 12-week LF-EMS intervention on several pro- and anti-inflammatory cytokines, in comparison with a 12-week IDC intervention group and a usual care control group.

Forty-eight HD patients were randomised to usual care (n = 16, 10 males, age 53.8 ± 17.3 years, height 1.68 ± 0.09 m, body mass 77.37 ± 18.00 kg (mean ± SD)), IDC (n = 15, 12 males, 52.0 ± 15.3 years, 1.71 ± 0.08 m, 85.87 ± 21.81 kg) or LF-EMS (n = 17, 14 males, 51.5 ± 17.8 years, 1.74 ± 0.07 m, 73.61 ± 12.24 kg) conditions. All patients completed a cardiopulmonary exercise test and had venous blood samples taken at baseline. The IDC and LF-EMS conditions then completed 12 weeks of progressive, moderate intensity, thrice-weekly intradialytic exercise training, whilst the control condition maintained their usual care routine. The cardiopulmonary exercise test and venous blood samples were repeated following the intervention. Pro- (IL-2, IL-6, IL-17a, TNF-α) and anti-inflammatory (IL-10) cytokines were analysed using a multiplex cytometric bead array technique. Statistical analysis was completed using IBM SPSS version 23.

Effect sizes are presented as η^2 (0.2 = small, 0.5 = moderate, 0.8 = large; Cohen 1988). Patients completed 93.0 ± 0.1 % of sessions in the IDC condition, and 91.0 ± 0.1 % in the LF-EMS condition. VO_2 peak and maximum power achieved in the cardiopulmonary exercise test were significantly improved in the IDC ($p = .002$ and $p = .001$, respectively) and LF-EMS ($p = .033$ and $p < .001$, respectively) conditions, however remained unchanged in the usual care condition ($p=.716$ and $p=.240$, respectively). Circulating

IL-2 and IL-17a concentrations were largely undetectable, and no significant group effects of the training interventions were observed in any other assessed cytokines (IL-6, IL-10, TNF-α) ($p \geq .059$, $\eta^2 \leq .097$).

Both the LF-EMS and IDC interventions were successful in improving measures of physical performance in these HD patients, however they did not significantly improve any of the measured circulating markers of inflammatory status. It is unclear why no changes were seen in inflammatory markers considering the significant effect of the exercise interventions on performance measures. However, possible explanations include changes in immune cell phenotypes, large differences at baseline between groups, large inter-patient variations caused by the heterogeneity of HD patients, or insufficient exercise intensity to elicit a change in resting inflammatory markers. However, significant alterations in resting circulating TNF-α were seen in Study 2 in response to the exercise intervention despite the average exercise intensity being significantly lower than seen here. This may be due to the number of TNF-α samples that were undetectable. It seems as though regular intradialytic LF-EMS training, whilst not eliciting an anti-inflammatory response, does not elicit a pro-inflammatory response and does improve exercise performance. Therefore, LF-EMS provides a viable alternative for patients with severe functional limitations that preclude the use of more traditional exercise modalities.

6.2 Introduction

Haemodialysis (HD) patients display elevated cardiovascular risk and all-cause mortality when compared to the general population (Methven, Steenkamp, and Fraser 2017), which can only be partly attributed to traditional risk factors (Longenecker 2002). As such, cardiovascular disease is the leading cause of death in this population, followed by infection (Methven et al. 2017). Traditional risk factors include hypertension, diabetes, dyslipidaemia and metabolic syndrome, whilst non-traditional risk factors include inflammation, oxidative stress and uraemia. As explained in Chapter 2, chronically elevated systemic inflammation as result of uraemia and the associated treatments can cause endothelial dysfunction and drive cardiovascular risk by eliciting oxidative stress (Bautista 2003) as well as by increasing leukocyte (primarily macrophage, T lymphocyte and mast cell) recruitment into the vasculature and accelerating atherosclerosis (Hansson 2003) and plaque destabilisation. HD patients also display impaired immune function (Girndt et al. 1999) and aberrant immune activation (e.g. increased Th1 stimulation, monocyte pre-activation and complement activation) which can promote inflammation via increased immune cell pro-inflammatory cytokine synthesis (Amore and Coppo 2002). Therefore, any intervention which can positively influence the cardiovascular system, enhance immune function and therefore reduce inflammation in HD patients would be expected to reduce both morbidity and mortality.

HD patients exhibit markedly reduced physical activity levels when compared to the healthy population (Avesani et al. 2012; Johansen et al. 2000), which results in detrimental changes in body composition (Johansen et al. 2003) and reduced functional capacity (Heiwe, Clyne, and Dahlgren 2003). A potential therapeutic strategy to diminish or reverse these negative changes is exercise. The beneficial effects of exercise on the cardiovascular system are well documented in both the general population (Shephard and Balady 1999) and the HD population (Deligiannis et al. 1999; Mustata et al. 2004). Exercise can also positively influence systemic inflammation in the CKD and HD populations via a reduction in pro-inflammatory mediators (Peres et al. 2015; Viana et al. 2014). However, HD patients may experience barriers to traditional exercise modalities, as explained below.

Whilst traditional aerobic and resistance training have been previously researched in the HD population (Chapter 2), some HD patients may experience many perceived barriers which may prevent their participation in traditional exercise interventions. These include lack of time, concerns over the safety of unsupervised exercise, lack of knowledge of how to exercise correctly and lack of access to facilities (Delgado and Johansen 2012). Intradialytic cycling (IDC) offers a solution to many of these problems as it is completed during normal treatment hours under the supervision of exercise professionals. Regular IDC has been shown to partially ameliorate cardiovascular risk (Toussaint et al. 2008), increase health-related quality of life (Ouzouni et al. 2009), and is also suggested to ameliorate systemic inflammation (by reducing pro-inflammatory mediators such as IFN-γ and intermediate monocytes and increasing IL-10 and regulatory T cells concentration) (Dungey et al. 2017; Peres et al. 2015). However, IDC may not be suitable for HD patients with contraindications to dynamic exercise including severe functional/orthopaedic impairments or cardiac instability.

Intradialytic, low frequency electrical muscle stimulation (LF-EMS) is a possible alternative strategy. In LF-EMS, electrodes placed on the skin deliver low-intensity electrical impulses to a specifically targeted muscle, which reacts by contracting just as it would in response to a physiological stimulus (Heidland et al. 2013). Therefore, LF-EMS can be used as an exercise mimetic in those who are unable to exercise volitionally, for instance due to musculoskeletal impairments or extreme frailty (though it must be noted that LF-EMS only mimics the local skeletal muscle contraction and does not incorporate many of the systemic mechanisms of volitional exercise (Maffiuletti et al. 2011)). The duration and intensity of stimulation can be altered to closely represent the standard progressive overload seen in traditional exercise interventions.

Preliminary evidence suggests that LF-EMS can be used to prevent intra- and post-HD hypotension, as well as potentially increase dialysis efficacy via increased toxin removal (Farese et al. 2008). LF-EMS can also mimic exercise more closely by increasing muscular strength and functional capacity in HD patients (Roxo et al. 2016; Simo et al. 2015), though has been shown to have no effect on endothelial function as assessed by brachial artery flow-mediated dilation (Schardong et al. 2017). However, there is very little

evidence investigating how LF-EMS affects systemic inflammation HD patients. Brüggemann et al investigated 'high' (50Hz, 72.90mA) and 'low' (5Hz, 13.85mA) intensity LF-EMS in HD patients (1 hour/HD treatment, 3 times/week for 4 weeks). They found that whilst both groups improved to a similar degree in quadriceps strength and 6 minute-walk distance, TNF-α was unchanged in both groups and IL-10 increased in the high intensity group only and (Brüggemann et al. 2017). Regular IDC has displayed the potential to attenuate systemic inflammation over a longer intervention (i.e. 8+ weeks) in this patient cohort (Afshar et al. 2010; Peres et al. 2015). Therefore, as the beneficial effects on physical function seen in LF-EMS are similar to those seen in IDC, a longer LF-EMS intervention could be expected to positively impact inflammation in a similar manner to IDC. This is because, as in other studies in this patient population, improvements in physical function are often paralleled by improvements in systemic inflammation (Afshar et al. 2010). Lastly, other pertinent inflammatory mediators (e.g. IL-6) have yet to be assessed in this regard.

Therefore, the aim of this study was to compare the effects of a 12-week progressive LF-EMS intervention protocol against both regular IDC training and standard HD care (i.e. no IDC or LF-EMS) on markers of systemic inflammation in HD patients. It is hypothesised that based upon the results of previous research and those obtained in study 2, that the IDC may reduce circulating TNF-α concentration when compared to standard HD care, and that this effect may also be seen in the LF-EMS condition but to a lesser degree as it is a more local exercise stimulus and therefore may not produce the same systemic effects as dynamic volitional exercise.

6.3 Methods

Ethics

A favourable ethical opinion was given by the West Midlands Research Ethics Committee (13/WM/0494) and patients provided written informed consent prior to participation.

Acknowledgement of secondary analysis

This study was largely completed by a research team based at the University Hospitals of Coventry and Warwickshire, under the supervision of Dr Gordon McGregor. As such, all of the information presented in the methods section of this study, aside from the cytokine and statistical analysis, was completed by Dr McGregor's research team and the full paper describing this methodology and primary outcomes has been published (Mcgregor et al. 2018). Following the collection of plasma samples during this study, the samples were delivered to Loughborough University, after which I was responsible for all laboratory analysis of cytokines as explained above, as well as all subsequent statistical analysis. Additionally, one patient that was included in the original study was deemed unsuitable for inclusion in the laboratory analysis due to the presence of a chronic infection that precluded the delivery of their blood samples to Loughborough).

Recruitment

Eligible patients were approached by a research nurse or investigator during their routine haemodialysis sessions, where they were given the patient information sheet and had the study protocol explained to them. Willing patients were consented to the study on their following haemodialysis session, provided they were suitable with regards to the inclusion and exclusion criteria:

Inclusion criteria:

1. On conventional, unit-based haemodialysis for at least 3months
2. On 3 times 4 hours of dialysis per week
3. Urea reduction rate of at least 65% during the three months before enrolment
4. Age 18 years or older

5. Able to complete the cardiopulmonary exercise test (CPET) and exercise training
6. Able to provide informed consent
7. Life expectancy of more than 6 months according to clinical assessment

Exclusion criteria:

1. Clinically significant valvular insufficiency
2. Clinically significant dysrhythmia
3. Uncontrolled blood pressure: systolic > 160 mmHg, diastolic >95 mmHg during the months before enrolment
4. Excessive fluid accumulation between dialysis sessions (>3 liters), pulmonary edema more than twice over 3 months before enrolment deemed to be due to excess fluid intake
5. Haemoglobin unstable and below 9.0 g/dL
6. Ischemic cardiac event or intervention in the last 3 months
7. Morbidly obese, mid-thigh circumference of more than 60cm (EMS straps limit)
8. Clinically significant, active inflammatory or malignant process
9. Pacemaker or cardiac device (contraindicated for bioelectrical impedance)
10. Patient highly physically active on their own accord
11. Planned kidney transplant during study period

Study Design

This study employed an open-labelled, randomised controlled study design. After recruitment, patients were randomised into one of three study groups; IDC, LF-EMS, or usual care. The study flow is outlined in Figure 6.1.

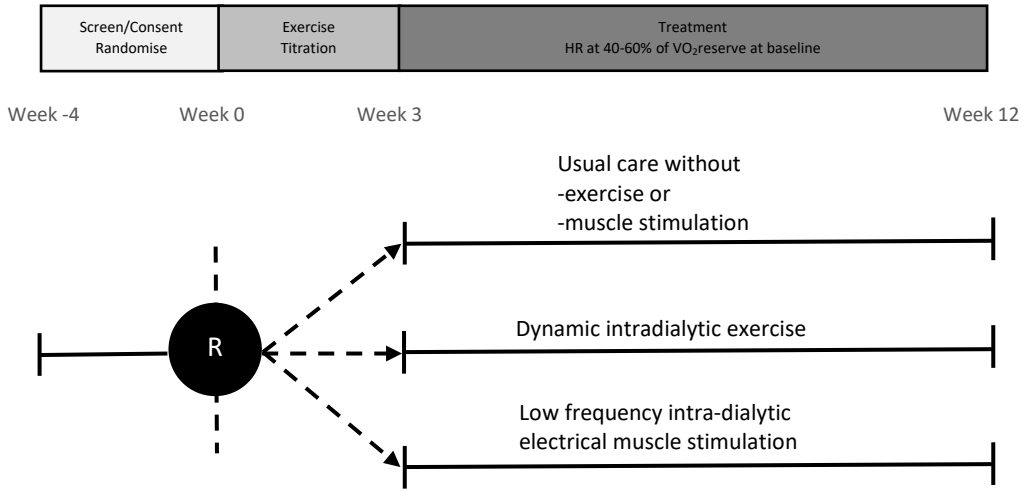

Figure 6.1. Study flow diagram. R = Randomisation

Randomisation visit (week 0)

This visit included a CPET to determine VO_2 peak, completed on a non-dialysis day at least 12 hours after the last dialysis session. A venous blood sample was also taken on this visit. Patients were then randomised to one of the three groups following this test (LF-EMS Training, IDC Training or Usual Care Control). Randomisation was accomplished using a stratified permuted block randomisation protocol with a variable block size (3 and 6). The stratification variables were age (under 55 years, 55 years or older) and sex (male, female). A separate permuted block randomisation was performed within each strata. The randomisation was competed at the University of Warwick medical school. The research team then contacted the statistician in order to obtain the allocation of each patient; however, the randomisation list was not made available to the study team.

Treatment – familiarisation period (week 0-3)

Patients allocated to either of the intervention groups completed a 3-week familiarisation period, to

allow a gradual build up to 30 mins of exercise or LF-EMS. Exercise workload and LF-EMS intensity were also gradually built up during this time, reaching the appropriate level of 40-60% VO_2 reserve (as assessed by their previous CPET) by the end of week 3. Rating of perceived exertion (RPE) was also used to monitor workload and intensity in both groups.

Graded LF-EMS test (week 3)

To ensure that LF-EMS tolerance had improved to a level that elicited a workload of 40-60% VO_2 reserve, a graded-LF-EMS test was performed following the 3-week familiarisation period. This was performed during a dialysis session. Stimulation intensity was incrementally increased every 5 minutes until the maximum tolerable level was achieved. Oxygen consumption was measured with a respiratory gas analysis system (Ultima CardiO2, Medical Graphics UK).

Intervention implementation (weeks 3-12)

Patients in the intervention groups progressed from 30 to 45 minutes of exercise at an intensity of 40-60% VO_2 reserve as regulated by heart rate and RPE (these varied depending on the patient however were based upon the heart rate and RPE values that were recorded during the CPET that equated to 40-60% VO_2 reserve). Where this wasn't possible, the maximal tolerated exercise intensity was continued.

Treatment completion visit (week 13)

Following completion of the intervention, patients came in on a non-dialysis day. Another blood sample was taken, and a CPET completed as above.

Cardiopulmonary exercise test

A CPET was completed on an electronically-braked cycle ergometer (Ergoselect 100, Ergoline) in combination with a breath-by-breath gas analyser (Ultima CardiO2, Medical Graphics UK) to measure O_2 uptake. Gas analyser and volume calibrations were completed before each test. Patients began cycling at 20 Watts, which was increased by 10-15 Watts/min, in order to reach volitional exhaustion in 9-12 mins. Patients were encouraged to maintain a cadence of 70 rpm until symptom limited volitional fatigue was reached, defined as a respiratory exchange ratio (RER) of >1.15. Breath-by-breath

respiratory gas exchange measurements of oxygen uptake (VO_2), carbon dioxide production (VCO_2) and minute ventilation (V_E) were recorded. Peak VO_2 was determined as the mean O_2 uptake in the final 20 seconds of the test.

Intradialytic exercise

Dynamic exercise was performed 3 times per week during the second hour of dialysis, either using a recumbent exercise bike (UBE-BD, Hudson Fitness) or a leg ergometer whilst seated in a dialysis chair. Following the familiarisation period (where exercise duration was progressively increased during the first 3 weeks), exercise intensity was gradually increased during the remaining 9 weeks of the intervention. If not already accomplished during familiarisation, the duration was also increased to 45 min, which then remained stable throughout. Each session was preceded and followed by a 5-minute warm-up and cool-down, respectively, at a self-selected pace.

Intradialytic LF-NMES

Bilateral neoprene straps were applied to the quadriceps and hamstrings (Figure 6.2) 3 times per week during the second hour of dialysis. Rhythmical contractions were applied at a frequency of 3-4Hz. Participants were introduced to the EMS protocol very gradually to ensure tolerance and adherence. On the first occasion the straps were activated for 5 minutes at a very light current amplitude (milliamps, mA) (i.e. less than visible contraction), so that the participants could become accustomed to the unusual sensation. During the following 8 familiarisation sessions (3 weeks), the duration was incrementally increased to 30 mins. Intensity was increased during this time as tolerated. The goal following this 3-week 'build-up' phase was to have each patient accustomed to using the straps for 30 mins at an intensity equivalent to 40-60% VO_2 reserve, as assessed by an intradialytic graded LF-EMS CPET, as described above. During LF-EMS sessions, exercise intensity was regulated using a combination of heart rate and rating of perceived exertion (RPE). Intensity was further increased over the following 9 weeks of the intervention to the maximum tolerated level, which varied on an individual basis. This was necessary to ensure that, whilst cardiovascular reserve and skeletal muscle function were improving, the intensity remained at a level that elicited a cardiovascular exercise response. Duration was also

increased to 45 mins by week 2 of the 9-week intervention, and remained at this level throughout. Each session was preceded by a 10 minute 'ramp-up' period during which intensity was gradually increased to the training level. Each session concluded with a 5-minute cool-down at a gradually reducing intensity. The LF-EMS units recorded the intensity and duration of the stimulation for each session, which was subsequently downloaded for analysis.

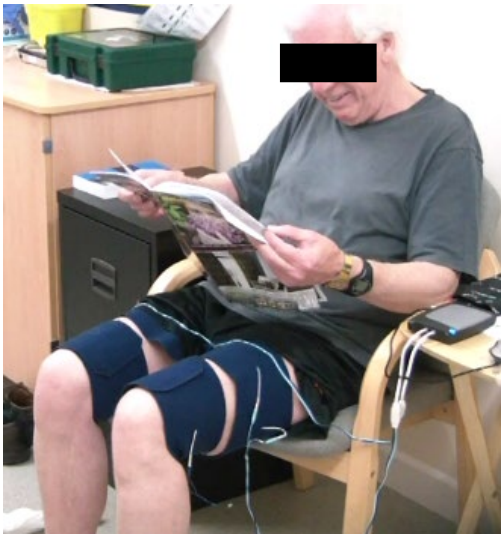

Figure 6.2. Demonstration of the positioning of the neoprene straps used to administer LF-EMS.

'Standard care' control group

Participants randomized into this group did not receive any form of intra-dialytic exercise treatment, and were not encouraged to participate in any interdialytic exercise on their own.

Cytokine analysis

Pro- and anti-inflammatory cytokines were assessed using a cytometric bead array – a detailed explanation of the principles and protocol of this procedure is included in Chapter 3. Interleukin-2 (FL2 human anti-IL-2), interleukin-6 (FL2 human anti-IL-6), interleukin-10 (FL2 human anti-IL-10), interleukin-17a (FL2 human anti-IL-17a) and tumor necrosis factor alpha (FL2 human anti-TNF-α) were analysed using an enhanced sensitivity flex-set, allowing the construction of a specific panel of analytes and providing a minimum detection threshold of 0.274 pg/mL for all analytes (as mentioned in previous studies, all samples that fell below this threshold were omitted from all analyses – the number of included samples is listed with each variable). Briefly, K_3EDTA-treated (1.6mg/mL blood) plasma was

thawed at room temperature and then incubated for 3 hours with beads which bind with the analyte of interest due to antibodies coated on their surface (specific to each analyte). These beads were then analysed using flow cytometry (BD Accuri C6, BD Biosciences, Oxford, UK), which allows the detection of each distinct bead population based on their pre-determined fluorescence intensity of a dye coated on their surface (the 'cluster parameter'). The degree to which each bead binds with its respective analyte is then proportional to the fluorescence intensity of the detection reagent and thus the analyte concentration ('reporter parameter'). This intensity for each analyte was then compared to its respective standard curve in a separate software (FCAP Array v. 3.0, BD Biosciences) which provides quantitative concentration results for all 5 cytokines within each sample.

Statistical analysis

Any non-normally distributed data (i.e. Shapiro-Wilks of $p<.05$) was logarithmically transformed to allow ensure assumptions of normality were met and therefore allow the use of parametric testing. All data was either normally distributed or successfully transformed – this is presented with each variable in the result section. Baseline differences were assessed using a one-way ANOVA - if a significant difference between groups was found, *post-hoc* testing was used to analyse multiple comparisons and the derived p-values were adjusted using the Bonferroni method (McHugh 2011). The effect of the intervention was assessed using a mixed-design ANOVA. In the case of one variable (circulating IL-17a concentration) data was only available for the LF-EMS condition and thus a paired samples t-test was used to compare pre to post-intervention values. Partial eta squared (η^2) was used to estimate effect sizes for all ANOVAs. Effect sizes were classified as small (0.2), medium (0.5) and large (0.8) (Cohen 1988). All descriptive data are presented as 'mean ± standard deviation' whilst outcome data is reported as 'mean ± standard error of the mean' when presented in a figure and 'mean ± SD' when presented in a table or discussed in the text. All statistical analysis was completed on IBM SPSS version 23.0 (Chicago, Illinois).

6.4 Results

Participants

Out of a total of 348 HD patients assessed for eligibility, 64 were deemed eligible and randomised to IDC (n = 20), LF-EMS (n = 22) and usual care control (n = 22). Due to changes in eligibility, missing samples and volitional withdrawal this resulted in 48 patients completing the study and being included in all laboratory analyses (IDC n = 15; LF-EMS n = 17; usual care n = 16). The Consolidated Standards of Reporting Trials (CONSORT) diagram illustrating the flow of recruitment and exclusion of patients is included in Figure 6.3.

The baseline demographic and clinical characteristics are included in Table 6.1.

Exercise Adherence and Adaptation

Exercise intervention adherence was good in both intervention groups, with 93.0 ± 0.1% of sessions being completed in the IDC group, and 91.0 ± 0.1% in the LF-EMS group. At the end of the intervention the mean duration and intensity of the exercise sessions was 56.3 ± 6.7 min and 63.8 ± 20.7 W in the IDC group, and 60.0 ± 0.1 min and 119.7 ± 13.0 mA in the LF-EMS group.

There was a significant time*group interaction effect for maximum load achieved in the CPET ($p < .001$, $\eta^2 = .316$). Post-hoc analysis indicated that max load increased from pre- to post-intervention in the IDC (100.1 ± 44.1 W to 116.3 ± 41.5 W; $p = .001$) and LFEMS (96.7 ± 25.5 W to 108.7 ± 28.1 W; $p < .001$) conditions but remained unchanged in the usual care condition (85.1 ± 26.1 W to 81.6 ± 28.7 W; $p = .240$). This is displayed in Figure 6.4a.

Similarly, there was also a significant time*group interaction effect for VO_2 peak ($p = .019$, $\eta^2 = .162$), as measured by the CPET. As with max load, VO_2 peak increased in the IDC (18.1 ± 6.7 mL/kg/min to 20.5 ± 7.0 ml/kg/min; $p = .002$) and LFEMS (19.7 ± 6.7 mL/kg/min to 21.0 ± 7.0 mL/kg/min; $p = .033$) conditions but remained unchanged in the usual care condition (16.7 ± 5.5 mL/kg/min to 16.5 ± 5.2 mL/kg/min; $p = .716$). This is displayed in Figure 6.4b.

	Usual Care (n = 16)	IDC (n = 15)	LF-EMS (n = 17)
Age (years)	53.8 ± 17.3	52.0 ± 15.3	51.5 ± 17.8
Male Gender, n (%)	10 (62.5)	12 (80.0)	14 (82.4)
Height (m)	1.68 ± 0.09	1.71 ± 0.08	1.74 ± 0.07
Weight (kg)	77.37 ± 18.00	85.87 ± 21.81	73.61 ± 12.24
BMI (kg/m^2)	27.22 ± 5.40	29.39 ± 7.69	24.31 ± 3.49
Systolic BP (mmHg)	119 ± 23	130 ± 27	117 ± 28
Diastolic BP (mmHg)	67 ± 15	73 ± 20	67 ± 14
Ethnicity, n (%)			
Caucasian	9 (56.3)	8 (53.3)	13 (76.4)
Asian	5 (31.3)	4 (26.7)	2 (11.8)
Black	2 (12.4)	3 (20.0)	2 (11.8)
Primary Diagnosis, n (%)			
Diabetic Nephropathy	2 (12.5)	4 (26.7)	0 (0)
Hypertensive Nephropathy	2 (12.5)	2 (13.3)	4 (23.5)
Pyelonephritis	1 (6.2)	1 (6.7)	4 (23.5)
Glomerulonephritis	5 (31. 2)	3 (20.0)	4 (23.5)
Other	3 (18.8)	1 (6.7)	4 (23.5)
Unknown	3 (18.8)	4 (26.7)	1 (6.0)
Comorbidities, n (%)			
Hypertension	12 (75.0)	12 (80.0)	13 (76.5)
Diabetes	5 (31.3)	7 (46.7)	12 (70.6)
Previous/Current Smoker	7 (43.8)	7 (46.7)	12 (70.6)
CVD	1 (6.2)	2 (13.3)	3 (17.6)
Clinical Laboratory			
Albumin (g/L)	44 ± 4	46 ± 4	45 ± 4
CRP (mg/L)	9 ± 10	5 ± 8	7 ± 7
Haemoglobin (g/L)	116 ± 13	111 ± 12	120 ± 10

Table 6.1. Baseline demographic and clinical data for all HD patients included in the analysis. Data is presented as mean ± SD. The number of decimal places presented in the mean is representative of the level of accuracy provided by the original measure. All data was normally distributed.

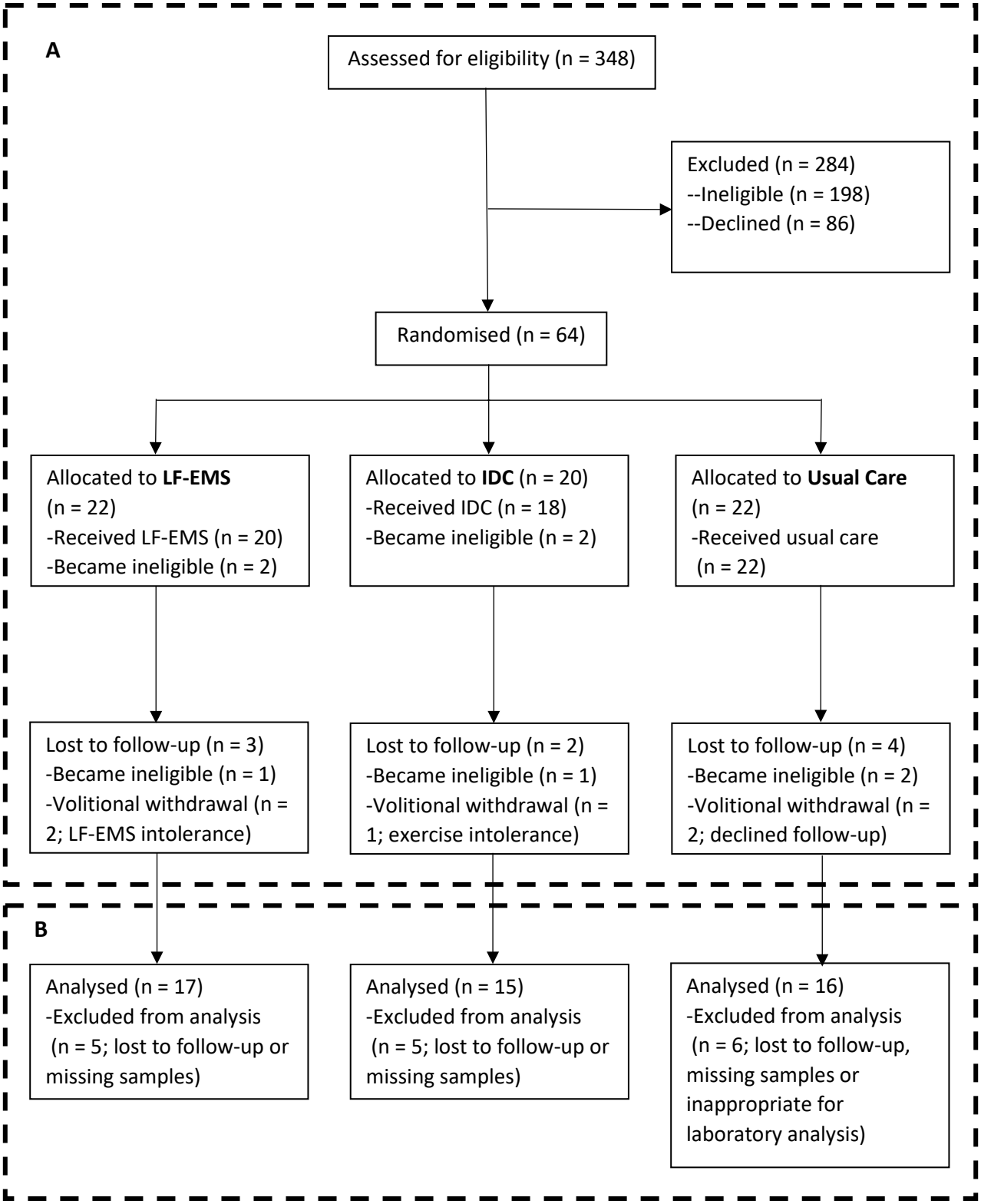

Figure 6.3. CONSORT diagram for the recruitment and analysis of HD patients. Dashed boxes are designed to acknowledge the work done at the University Hospitals of Coventry and Warwickshire (UHCW) trust by Dr Gordon McGregor and his research team. A = work completed externally at UHCW. B = work completed internally at Loughborough University.

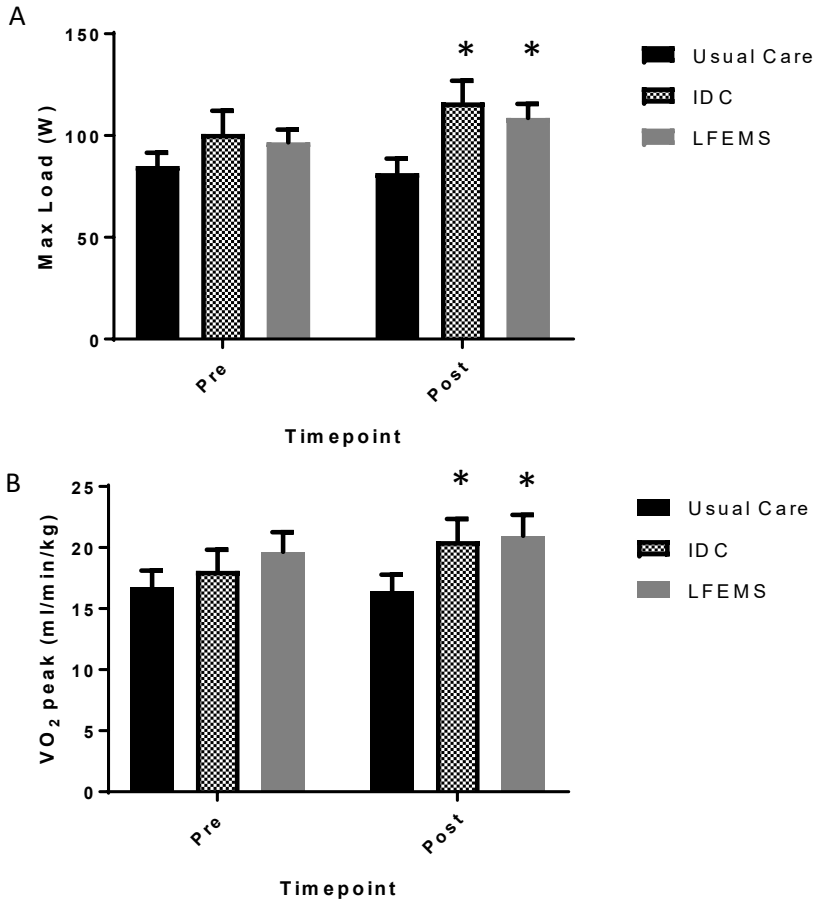

Figure 6.4. Demonstration of the changes that occurred in Maximum Load (A) and VO_2 peak (B) as assessed by the CPET in all three groups. * = significantly different from baseline. This data was normally distributed (control n = 16, IDC n = 15, LF-EMS n = 17). Data is presented as 'mean ± SEM'.

Cytokine results

IL-2

As was seen in study 2, all of the values obtained for circulating IL-2 concentration fell below the minimum detectable threshold (0.274 pg/mL) and thus were indistinguishable from background noise. As such, no valid data was obtained for IL-2 in this study and therefore none is presented.

IL-6

All included analysed samples (n = 48) provided valid measures of circulating IL-6 above the minimum detectable threshold. There were no significant differences between groups at baseline (p = .105) and no significant time*group interaction effect was exhibited (p = .146, η^2 = .082). Post-hoc sample size calculation suggested that a sample size of n = 63 would be required to elicit a significant interaction effect for circulating IL-6 levels. These results are displayed in Figure 6.5.

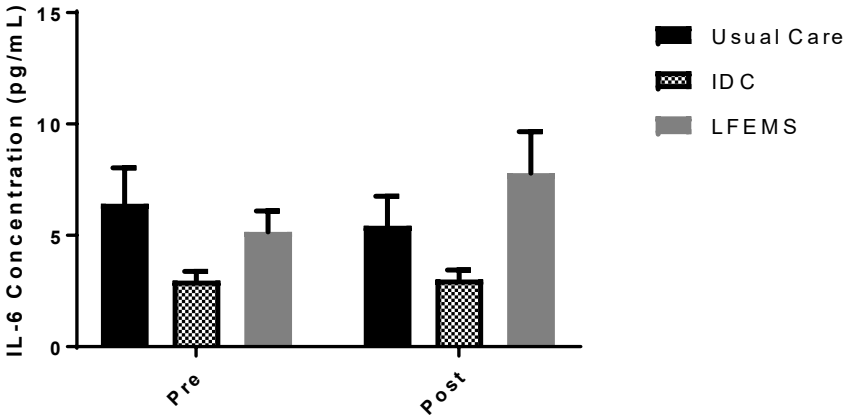

Figure 6.5. IL-6 levels, pre- and post-intervention in all three conditions. This data was normally distributed. (control n = 16, IDC n = 15, LF-EMS n = 17). Data is presented as 'mean ± SEM'.

IL-10

All included analysed samples (n = 48) provided valid measures of circulating IL-10 above the minimum detectable threshold. As with IL-6, there were no significant differences at baseline (p = .302) and no time*group interaction effect was observed (p = .438, η^2 = .036). Post-hoc sample size calculation suggested that a sample size of n = 108 would be required to elicit detectable significant findings for circulating IL-10 levels (interaction effect). These results are displayed in Figure 6.6.

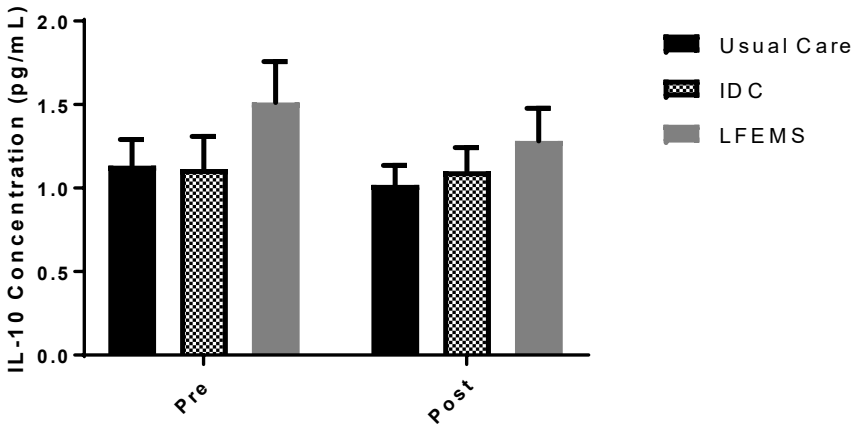

Figure 6.6. IL-10 levels, pre- and post-intervention in all three conditions. This data was normally distributed. (control n = 16, IDC n = 15, LF-EMS n = 17). Data is presented as 'mean ± SEM'.

IL-17a

Similar to Study 2, many of the IL-17a results in this study fell below the minimum detectable threshold and thus were removed prior to statistical analysis. Only 6 samples, all within the LF-EMS condition, returned valid results. As such, the effect of LF-EMS on circulating IL-17a levels was assessed with a pre-post paired samples t-test. However, these results should be interpreted with caution given the small sample size and lack of analysable control conditions. No significant differences were seen between pre and post-intervention values in circulating IL-17a values (p = .181). This is presented in Figure 6.7.

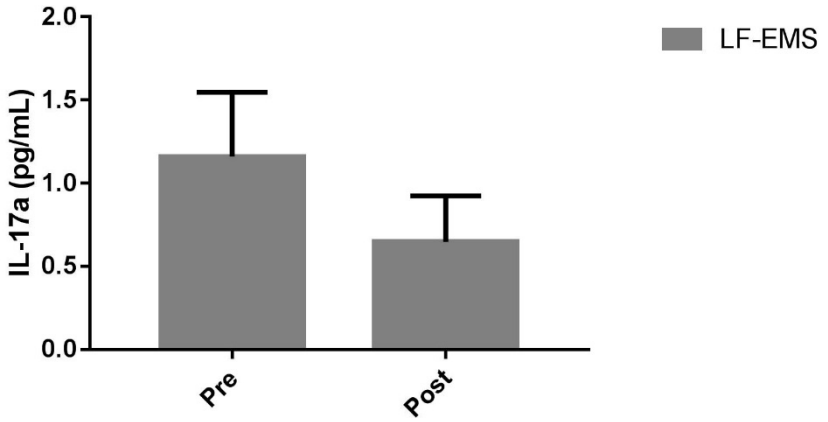

Figure 6.7. IL-17a levels, pre- and post-intervention in all three conditions. This data was non-normally distributed and thus logarithmically transformed prior to analysis. (LF-EMS n = 6). Data is presented as 'mean ± SEM'.

TNF-α

In a similar manner to study 2, a number of the analysed samples displayed TNF-α values that were below the minimum detectable threshold. After discarding these results, valid TNF-α concentration data was available for a total of 23 patients (control n = 7, IDC n = 7, LF-EMS n = 9) and as such these results should be interpreted with some caution given the reduced sample size. No group differences were observed at baseline (p = .059). Additionally, no time*group interaction effect was observed (p = .672, η^2 = .039). Post-hoc sample size calculation suggested that a sample size of n = 99 would be required to elicit a significant interaction effect for circulating TNF-α levels. These results are presented in Figure 6.8.

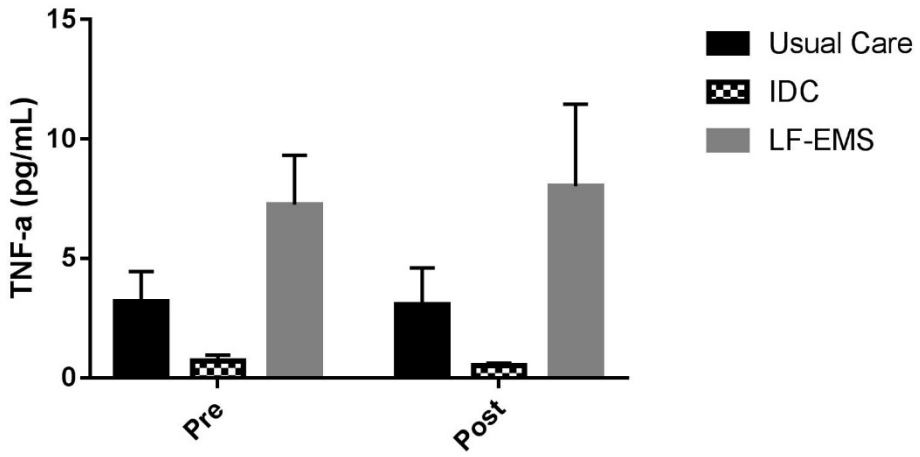

Figure 6.8.TNF-α levels, pre- and post-intervention in all three conditions. This data was non-normally distributed and as such was logarithmically transformed prior to analysis. (control n = 7, IDC n = 7, LF-EMS n = 9). Data is presented as 'mean ± SEM'.

IL-6/IL-10 Ratio

To calculate the ratio of pro- and anti-inflammatory cytokines, IL-6/IL-10 ratio was calculated simply by dividing the former by the latter. This marker has been used previously as a surrogate for overall inflammatory 'balance' in CKD and has proven to be reactive to aerobic exercise (Viana et al. 2014). No between group differences were observed at baseline (p = .150). Similarly, no time*group interaction effect was observed (p = .232, η^2 = .063). Post-hoc sample size calculation suggested that a sample size

of n = 63 would be required to elicit detectable significant findings for circulating IL-6/IL-10 ratio (interaction effect). These results are displayed in Figure 6.9.

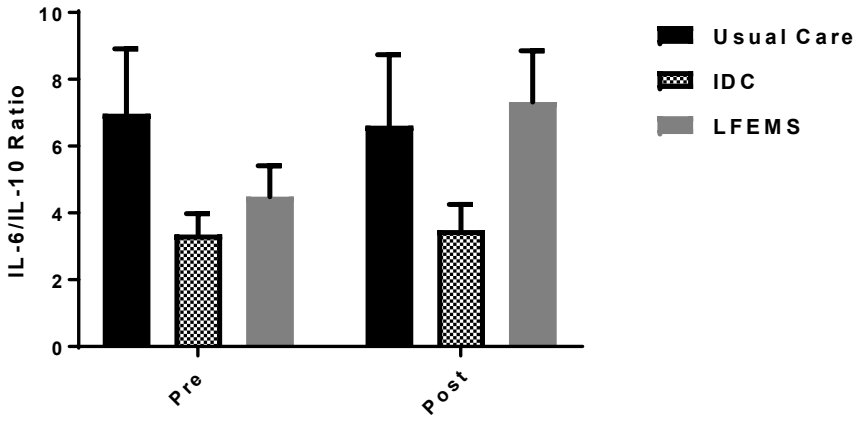

Figure 6.9. IL-6/IL-10 ratios, pre- and post-intervention in all three conditions. This data was non-normally distributed and thus was logarithmically transformed prior to analysis (control n = 16, IDC n = 15, LF-EMS n = 17). Data is presented as 'mean ± SEM'.

In summary, all of the circulating IL-2 values fell below the minimum detectable threshold of the cytometric bead array technique, as well as the majority of the IL-17a values and roughly 50% of the TNF-α values. In those cytokines that provided valid and measurable values. Regardless, there were no significant effects of any of the three conditions on any of the cytokines measured in this study, or on the ratios between the cytokines.

6.5 Discussion

The aim of this study was to investigate the effects of a regular LF-EMS intradialytic training programme on soluble markers of inflammation and compare this to the effects of a more conventional IDC training programme. Both training modalities increased aerobic performance and maximum power output, whilst no significant effects were seen in the usual care control group. However, there were no discernible effects on inflammatory markers in any of the groups.

Adherence

Exercise intervention adherence in both treatment groups was excellent, with patients in both conditions completing an average of over 90% of the possible sessions. This compliance is generally higher than those of other training intervention studies in ESRD patients completed outside of dialysis (Mustata et al. 2004; Smart and Steele 2011). As shown previously (Konstantinidou et al. 2002) intradialytic exercise is effective in providing high intervention adherence as it does not require extra time from the patient beyond that which they already give to dialysis treatment and therefore this high adherence rate was to be expected.

As a result of the high exercise adherence, by the end of the intervention the IDC group were achieving a mean session duration of 56.3 ± 6.7 min and a mean session power of 63.8 ± 20.7 W. This is generally higher than those seen in previous IDC interventions (Bohm et al. 2014; Dungey et al. 2017). Likewise, LF-EMS patients achieved a mean session duration of 60.0 ± 0.1 min and a mean session intensity of 119.7 ± 13.0 mA. However, there is less data available from previous research to compare these values to as several intradialytic LF-EMS training studies do not report average session intensity (Roxo et al. 2016; Schardong et al. 2017; Simo et al. 2015). Similarly, studies which have investigated LF-EMS in other conditions (such as COPD or patients in intensive care units) have also failed to report either mean session intensity or a progression in session intensity (Bourjeily-Habr et al. 2002; Karatzanos et al. 2012).

Physical performance

Patients from both the IDC and LF-EMS groups demonstrated significant improvements in markers of physical performance, whilst no change was seen in the control group. As was expected, patients in both exercise groups demonstrated a significant increase in both VO_2 peak and maximum load reached during the progressive CPET. This demonstrates the efficacy of both the more traditional exercise programme (IDC) and the more novel protocol (LF-EMS) in increasing aerobic fitness in HD patients. It also highlights their potential therapeutic strategy, as VO_2 peak has been found to be a strong predictor of survival in ESRD patients, even greater so than more traditional predictors of mortality in this population such as age, HD vintage and pulse pressure (Sietsema et al. 2004). The significant increase in VO_2 peak seen in the LF-EMS condition is particularly encouraging, as it may offer patients who would otherwise be unable to complete traditional exercise regimes (for instance due to severe functional limitations) an alternative strategy through which they can increase their overall health. Future research should focus on identifying these patients and investigating the effects of LF-EMS in this cohort. However, it may be difficult to complete a similar study in these patients as by their nature they may be unable to complete traditional functional exercise testing.

Cytokine results

The cytokine results obtained during this study are characterised by a clear and uniform lack of changes in all treatment groups, most likely caused by the large inter-patient variation and the large differences seen between groups at baseline. However, it is still pertinent to discuss these cytokines, including how they may contribute to the systemic cytokine environment in this patient population and implications for future research.

IL-2 proved to be completely undetectable in all samples included in this study. IL-2 is a pro-inflammatory cytokine produced mainly by $CD4^+$ T lymphocytes. It also promotes T and B lymphocyte proliferation following antigen presentation, stimulates natural killer cell phagocytic activity and stimulates IFN-γ release by T lymphocytes. Previous research in non-ESRD healthy populations has shown that 12 weeks of regular (4-5 times/week for 12 weeks) moderate intensity (65-70% VO_2 max for

30 min) cycle ergometer training elicited a 33% increase in soluble IL-2 receptor levels whilst having no significant effect on circulating IL-2 levels. However, the training programme did attenuate the acute decrease in IL-2 levels seen after 60 min of submaximal exercise before the intervention (Rhind et al. 1996). A different study found that after 6 weeks of moderate intensity aerobic dance exercise (50 min, 3 times/week) in previously sedentary women, resting IL-2 levels were significantly increased when compared to baseline (Leelarungrayub et al. 2011). The authors suggest that this may represent an enhancement in immune function, as increased IL-2 levels would help to maintain or restore naïve T cell pools due to increased post-activation proliferation. Whilst no changes were seen in this study with regards to IL-2, there was a trend towards an increase in both the IDC and LF-EMS groups whilst the control group showed little change. However, it is unclear if an increase in IL-2 levels in ESRD patients would represent an improvement in immune function. An increase would certainly help to restore the naïve T lymphocyte pool as it would increase post-antigen presentation proliferation and thus increase overall numbers – this may help to reverse the increase in CD8/CD4 ratio that characterises the 'terminally aged' immune system of ESRD patients. It may also help to combat the poor vaccination responses seen in ESRD patients by improving post-vaccination seroconversion rates and thus eliciting a more potent B lymphocyte response (Cordova et al. 2017). However, immune cells in ESRD are also aberrantly and continuously activated by uraemic toxin ligation, and therefore it is unclear whether a rise in the concentration of a protein that increases cellular activation would be helpful or harmful, as it is primarily chronically activated immune cells that perpetuate the pro-inflammatory environment. However, as IL-2 levels were undetectable in this patient cohort, who would be presumed to display a chronically activated immune system, the clinical relevance of IL-2 must be called into question given its low circulating concentrations observed in this study. This may point towards a limitation of the cytometric bead array technique, and possibly suggests that a more targeted assay (e.g. ELISA) designed specifically to detect IL-2 may be more appropriate for future research.

IL-6 is one of the most frequently investigated inflammatory interleukins and represents an interesting target as it can have both pro- and anti-inflammatory effects depending on the cause and mechanism of release. When acting as a pro-inflammatory cytokine, IL-6 promotes lymphocyte proliferation, leukocyte

recruitment and induces an acute phase response in the liver, resulting in increased CRP (a potent pro-inflammatory positive acute phase reactant) production. IL-6 is produced mainly by activated monocytes and lymphocytes, and operates in an endocrine manner within the circulation (Stenvinkel et al. 2005). Conversely, when acting as an anti-inflammatory myokine, IL-6 is released from myocytes within the working muscle, causing a short-lived increase in the circulation which then stimulates a more long-lasting increase in circulating IL-1ra and IL-10 concentrations, both of which have anti-inflammatory properties (Pedersen and Febbraio 2008). In a small randomised crossover trial in 9 HD patients, 20 min of moderate intensity IDC did not impact circulating IL-6 levels (Peres et al. 2015), however a similar study (n = 10) found that an acute bout of IDC prevented the increase in circulating IL-6 that was seen in the non-exercise control condition (Wong et al. 2017). The HD process *per se* would most likely elicit increased IL-6 levels by increasing ligation and activation of immune cells resulting in increased secretion. However, conversely, HD removes uraemic toxins which also activate immune cells. Therefore, the impact of HD on immune cell activation and the additional influence of aerobic exercise requires investigation in larger sample sizes. Similar to previous results (Dungey et al. 2017), this study found no impact of an intradialytic training programme on IL-6 levels. A possible cause for this, beyond methodological limitations, is the limited muscle mass and relatively low exercise session intensity. HD patients display accelerated muscle wasting and sarcopenia (Chen et al. 2013; Honda et al. 2007) – as such the volume and degree of skeletal muscle activation accomplished during intra-dialytic exercise may simply not be great enough to elicit a significant rise in IL-6 secretion.

IL-10 is a cytokine with anti-inflammatory properties and is primarily released by regulatory T lymphocytes. It exerts its anti-inflammatory effects by restricting antigen presentation to immune cells, thus preventing immune cell activation and synthesis of pro-inflammatory cytokines. IL-10 can also inhibit atherogenesis by down-regulating adhesion molecule (e.g. ICAM-1) expression, preventing chemokine (e.g. IL-8) secretion and reducing the production of matrix metalloproteinase (Stenvinkel et al. 2005). This results in reduced adhesion of immune cells to the endothelium and reduced plaque destabilisation within the intima and media of the vascular wall. In non-ESRD patient populations (e.g. coronary artery disease, Type II diabetes) regular moderate intensity aerobic exercise training has been

associated with increased IL-10 levels (Goldhammer et al. 2005; Kadoglou et al. 2007). Similarly, moderate intensity walking exercise has been shown to increase IL-10 levels both acutely post-exercise and at rest following a 6-month home-based walking training programme which contributed to an increased IL-10/IL-6 ratio (Viana et al. 2014). Acutely, IDC has also been shown to increase post-exercise circulating IL-10 levels in HD patients (Peres et al. 2015), however the IL-10 response to regular IDC training has not yet been investigated to the author's knowledge. As a primary mechanism via which IL-10 is increased is in response to an increase in IL-6 secretion and IL-6 remained unchanged in this study, it is unsurprising that there was no subsequent IL-10 response. However, Dungey et al found that, following 6-months of thrice-weekly moderate intensity IDC, circulating $CD4^+CD25^+CD127^-$ T-reg numbers were decreased in the control group but not the exercising group (n = 16) (Dungey et al. 2017). As IL-10 is primarily produced by T-regs, it would be expected that this would result in increased circulating IL-10 levels relative to the control condition. However, it is also possible that the increase was as a result of an increase in the total circulating $CD4^+$ lymphocyte pool rather than specifically T-regs. Regardless, IL-10 was not quantified so it is hard to draw conclusions concerning the overall anti-inflammatory effect. A possibility for future research would be to investigate both circulating T-reg numbers and IL-10 levels in response to an IDC training programme.

Similarly to IL-2, IL-17a was largely undetectable and only provided valid results for 6 patients, all of whom were in the LF-EMS condition. This greatly limits the validity of this analysis and it is therefore unsurprising, given the small sample size, the lack of groups for comparison and the fact that no changes were observed in other circulating cytokine results, that no significant changes were observed in response to the intervention. IL-17a is a primarily pro-inflammatory cytokine produced mainly by Th17 lymphocytes. Aside from its role in host defence against extracellular bacteria and fungi, an elevated IL-17a concentration promotes many autoimmune and inflammatory conditions including asthma, multiple sclerosis and arthritis. It operates by eliciting secretion of neutrophil chemokines (e.g. IL-8), promoting immune cell secretion of IL-6 and TNF-α and stimulating complement activation (Cortvrindt et al. 2017). In a recent study investigating acute bouts of aerobic exercise of 2 different intensities (50% and 90% of max power achieved in a ramp test) on circulating inflammatory markers in

a non-ESRD population, IL-17a was unchanged following exercise in either condition (Dorneles et al. 2015). Aside from this study, the effects of exercise on both IL-17a and Th17 cells remains chronically under-researched, meaning there is little research to compare these results to. Both acute aerobic exercise and chronic aerobic exercise training can have profound effects on lymphocyte subset distributions, so it can be expected that Th17 cells and subsequently IL-17a would also be affected. However, again as with IL-2, the clinical relevance of a cytokine which largely displays such low circulating values must be questioned, especially since the more 'traditionally measured' cytokines (e.g. IL-6, IL-10) display circulating concentrations that are much higher. Further research should include this cytokine when investigating panels of inflammatory markers, given its potential to propagate a pro-inflammatory environment and promote autoimmune dysfunction. However, a more targeted and sensitive assay may be required to detect this cytokine and comment on its applicability to the HD population.

As was seen in study 2, circulating TNF-α was detectable in roughly half (23 out of 48) of the included patients. TNF-α is a pro-inflammatory cytokine which is predominantly produced by activated macrophages. Its primary function is immune cell regulation, including inducing apoptosis in tumor cells, promoting macrophage phagocytic activity and inducing cytokine release by leukocytes. TNF-α is elevated in ESRD patients chiefly due to reduced renal clearance, but also due to over-activation of immune cells (Stenvinkel et al. 2005), as with other pro-inflammatory cytokines. In a non-ESRD overweight population, 7 months of regular aerobic exercise (30-60 min/day, 70% HR reserve, 4-5 days/week) significantly reduced resting TNF-α levels (Kondo, Kobayashi, and Murakami 2006). However Peres et al found that, following a 20 min bout of moderate intensity IDC, there was only a trend towards a reduction in TNF-α levels (Peres et al. 2015) and Dungey et al found that 6 months of regular IDC training did not affect resting TNF-α levels (Dungey et al. 2017). One of the proposed mechanisms via which aerobic exercise training promotes an anti-inflammatory environment is by inducing a phenotype switch in macrophages from the more pro-inflammatory M1 type to the more anti-inflammatory M2 type (Gleeson et al. 2011), resulting in reduced TNF-α secretion into the circulation. Similarly, a shift from the more inflammatory intermediate and non-classical monocyte subsets to the

more anti-inflammatory classical monocyte subset is proposed to occur in response to aerobic exercise training (Shantsila et al. 2012). Therefore, when investigating the effects of aerobic exercise on resting TNF-α levels, it would be pertinent to also characterise the tissue-resident macrophage and circulating monocyte populations in order to investigate whether a subset shift has taken place. The lack of a significant change of TNF-α levels in this study would suggest that either there was not a sufficient shift towards the M2 phenotype/non-classical monocyte subset or that other mechanisms are influencing TNF-α levels, which would require further investigation. Additionally, it may also be a result of the relatively low number of detectable samples. Similar to the IL-10 findings, it may be pertinent in future studies to assess both the cytokine itself and the cellular source in order to more clearly elucidate the relationship.

Limitations

A clear limitation to this study is the inability of the cytometric bead array technique to detect the circulating concentrations of IL-2 and IL-17a (and to an extent TNF-α). However, given that the majority of the values of IL-2 and IL-17a were so low, it may be that these cytokines are less clinically relevant than IL-6 and IL-10, which displayed much higher circulating concentrations. Additionally, the coefficient of variation of the cytokine analysis technique was large (10.08%), thus increasing the risk of committing a type 2 error. Concerning the sample size, this study was relatively large when compared to previous IDC studies (Dungey et al. 2017; Peres et al. 2015; Wong et al. 2017). However, cytokine analysis was not the primary outcome measure of this study as a whole, and such the sample size necessary to provide adequate statistical power was not based on previous cytokine findings. As such, a larger sample size may be required in order to detect possible changes in circulating cytokine levels, as is demonstrated by the post-hoc power calculations.

Another possible limitation is the mean exercise session intensity achieved in this study. Despite being relatively high compared to other IDC studies (at least in the cycling condition), and much higher than the mean session intensity observed in Study 2, the exercise intensity was seemingly not great enough to elicit significant alterations in the inflammatory cytokines assessed here. However, the main aim of

this study was to investigate LF-EMS, and there are no reported mean session intensities in previous LF-EMS literature to which the results here can be compared. A possible way to investigate this would be to also include blood samples before and after a single dialysis session and before and after an IDC session (with a non-IDC control trial – as has been completed previously (Peres et al. 2015)) and combine this with resting samples taken longitudinally before and after a training programme. This would help to determine if a single LF-EMS session is sufficient to elicit an inflammatory response that is different (or distinguishable) from the previously reported (Chapter 2) acute inflammatory influence of HD treatment, and whether or not this relationship changes after regular LF-EMS training. This would then provide information as to whether or not a change in resting circulating cytokine levels could be expected following a longitudinal intervention. However, due to the time and labour intensive nature of sample collection, processing and analysis, only resting samples were collected in this study. To the author's knowledge, no intradialytic exercise (IDC or otherwise) study has completed inflammatory marker assessment both before and after an acute exercise session and during a regular training programme.

Conclusions and future research

In summary, neither the IDC nor the LF-EMS interventions impacted circulating inflammatory markers in these HD patients. However, both interventions were successful in improving measures of physical performance. This improvement is encouraging, particularly in the LF-EMS condition, as it may provide an avenue for certain HD patients to exercise who were previously unable to do so when implementing a more traditional exercise modality. Future research should focus on these patients to assess the feasibility of this more novel exercise modality in such a functionally limited group. When considering the cytokine results, future research should focus on first investigating the impact of an acute intradialytic LF-EMS session in order to characterise how this impacts circulating cytokine levels, followed by a longitudinal study design (if significant findings are observed) in order to investigate how any observed effects affect resting circulating cytokine levels when repeated over the course of a regular training programme. Similarly, the combination of cytokine and immune cell analysis within a

single study would provide a clearer picture of the cellular mechanisms in response to an exercise training programme. Lastly, larger sample sizes may be necessary to investigate the impact of regular LF-EMS on circulating cytokine levels, as the post-hoc sample size calculations suggested that a sample size of at least n = 63 would be required to observe significant alterations in response to the intervention.

Chapter 7

Study 4

The influence of moderate aerobic exercise on markers of immune function and inflammation in renal transplant recipients

7.1 Abstract

Renal transplant recipients (RTRs) exhibit increased cardiovascular risk, elevated levels of markers of chronic systemic inflammation and elevated circulating microparticle (MP) counts in a similar manner to the HD patient population. Additionally, RTRs must take immunosuppressive medication in order to prevent allograft rejection, which impairs immune function and increases susceptibility to infection. In the general and HD populations, moderate intensity aerobic exercise can decrease cardiovascular risk, alleviate chronic systemic inflammation, reduce circulating pro-thrombotic MP concentration and improve immune cell function. However, it is unknown how aerobic exercise affects these relationships in RTRs. This study investigated the effects of a single 20 min bout of moderate intensity walking exercise on immune cell phenotypes and circulating cytokines, chemokines and MPs in a RTR population, in comparison with a non-dialysis dependent chronic kidney disease (CKD) population (to act as a non-immunosuppressed, uraemic control group) and a healthy control (HC) population (to act as a non-immunosuppressed, non-uraemic control group).

Fifteen RTRs (13 males, age 52.8 ± 14.5 years, height 1.70 ± 0.08 m, body mass 73.14 ± 14.05 k; 'mean ± SD'), 16 CKD patients (8 males, 54.8 ± 16.3 years, 1.67 ± 0.13 m, 78.53 ± 17.55 kg) and 16 HCs (9 males, 52.2 ± 16.2 years, 1.71 ± 0.11 m, 73.51 ± 13.50 kg) completed 20 minutes of moderate intensity (estimated 85% of VO_2 peak) walking exercise. Venous blood samples were taken at baseline, immediately after exercise and 1-hour after exercise cessation. Immune cell and MP phenotypes were assessed by flow cytometry, and pro- and anti-inflammatory cytokine and chemokine concentrations were assessed by a cytometric bead array multiplex technique.

The exercise bout elicited an increase in the proportion of circulating classical and non-classical monocyte subsets and a decrease in the proportion of circulating intermediate monocytes in all groups ($p \leq .002$, $\eta^2 \geq .103$). RTRs displayed a greater percentage of monocytes expressing angiotensin-converting enzyme (ACE) ($p \leq .001$), an elevated MIG (monokine induced by gamma interferon) concentration ($p \leq .005$), and a trend towards an elevated MCP-1 (monocyte chemotactic protein) concentration ($p \leq .052$) compared to the other two groups, whilst the HCs displayed an elevated

regulatory T cell number ($p \leq .022$). However, the exercise bout did not significantly impact these relationships. Lastly, little differences were seen between groups regarding MP phenotypes, however the exercise bout induced a decrease in TF expression on platelet-derived MPs ($p = .010$, $\eta^2 = .229$) in all groups, re-enforcing the similar findings observed in Study 1.

The exercise-induced reduction in intermediate monocyte percentage in all groups is encouraging, given the previously observed associated between this subset and cardiovascular events in the CKD and ESRD populations. Whilst T cell and monocyte-specific chemokines (MIG and MCP-1) may be elevated in RTRs in response to an immunosuppression-induced reduction in cell motility, there were no differences seen between groups in circulating cytokine levels and the exercise bout did not impact these relationships in any of the groups. Lastly, the exercise bout elicited a reduction in TF expression on neutrophil MPs in all groups. The reduction in the percentage of TF-positive platelet-derived MPs is encouraging and suggests a possible exercise-induced reduction in thrombotic potential – further functional assays should be completed to investigate this. Most importantly, the impact of the exercise bout did not differ between the groups and the exercise did not cause aberrant immune system activation or inflammation in the RTRs, meaning moderate intensity exercise participation can be considered immunologically safe and should be encouraged just as with the general population. Further research should investigate other exercise intensities and modalities in this patient population.

7.2 Introduction

Chronic Kidney Disease (CKD) patients display elevated cardiovascular disease (CVD) risk alongside reduced habitual physical activity levels and detrimental changes in body composition (reduced skeletal muscle mass, increased body fat mass) (Beddhu et al. 2009; Johansen and Lee 2015; Subbiah et al. 2016). However, traditional risk factors such as obesity, hypertension or hypercholesterolaemia only partly explain this increased cardiovascular risk (Shlipak et al. 2005). Infection currently accounts for 18-24% of deaths in CKD patients in the UK (Methven et al. 2017). CKD patients also display chronically elevated levels of systemic inflammation (e.g. elevated IL-6 and TNF-α, reduced IL-10), which can increase chemokine (e.g. MCP-1, RANTES, IL-8) and adhesion molecule (e.g. VCAM-1, ICAM-1) expression, thus promoting leukocyte tethering and extravasation into the vasculature and driving atherosclerosis (Akchurin and Kaskel 2015; Hansson 2003; Steyers and Miller 2014) and increasing CVD-related mortality (Abedini et al. 2009). Chronically elevated systemic inflammation may be caused by reduced renal clearance of cytokines, or uraemic toxin build-up due to reduced renal clearance (Castillo-Rodríguez et al. 2017). This uraemic toxin build-up may also cause chronic immune activation resulting in immune impairment, characterised by increased leukocyte apoptosis, impaired inhibitory activity of regulatory T cells (T-regs), expansion of pro-inflammatory ($CD16^+$) monocyte subsets, reduction in CD4/CD8 T cell ratio and depletion of naive T cells, B cell lymphopenia, increased monocyte cytokine production and increased spontaneous granulocyte ROS production (Neale et al. 2015; Vaziri et al. 2012). In renal transplant recipients (RTRs), this immune impairment is exacerbated by the immunosuppressive medication necessary to prevent allograft rejection (Hutchinson et al. 2003), which dampens the T lymphocyte proliferative response to antigen presentation, as well as inhibiting overall T lymphocyte function. Consequentially, the combination of the uraemic milieu and immunosuppressive regime leaves the leukocytes of RTRs chronically activated, resulting in elevated resting cytokine production, yet functionally anergic resulting in an impaired ability to combat infections.

Microparticles (MPs) are small (0.1-1.0 μm) extracellular vesicles shed upon cellular activation or apoptosis and can act as biomarkers for leukocyte dysfunction. MPs also have pro-inflammatory and pro-thrombotic bioeffector functions via the binding of TF with the phosphatidylserine exposed on their

outer surface (Ando et al. 2002; Del Conde et al. 2005; Piccin et al. 2007). Circulating MP numbers are elevated in CKD and renal failure patients (Daniel et al. 2008; Dursun et al. 2009; Faure et al. 2006) but may be reduced following renal transplantation (Al-Massarani et al. 2009); however not to levels seen in the general population. As such, MPs may be another mechanism behind increased CV risk in RTRs, as well as a driver of inflammation.

A potential contributory factor for the increased systemic inflammation in RTRs, aside from those mentioned above, is reduced skeletal muscle mass and increased adipose tissue mass caused by both reduced physical activity levels and inflammation-induced skeletal muscle protein breakdown (Costamagna et al. 2015). Pedersen et al demonstrated that skeletal muscle can act as an endocrine organ releasing IL-6 and thus can impact systemic inflammation (Pedersen and Febbraio 2008). Similarly, adipose tissue has been shown to be an active tissue with regards to systemic inflammation, and the pro-inflammatory adipokine adiponectin has been correlated with a variety of pro-inflammatory cytokines in the CKD population (IL-6, IL-18, TNF-α) (Lee et al. 2010). Additionally, whilst the immunosuppressive regime is necessary to avoid rejection, it also negatively impacts body composition, causing corticosteroid-induced weight gain (Lentine et al. 2008) and muscle wasting (Horber et al. 1986). Therefore, RTRs are at the mercy of several other causes of systemic inflammation, beyond solely aberrant immune cell activation. CKD patients also exhibit increased monocyte angiotensin-converting enzyme (ACE) expression, contributing to their increased CVD risk (Ulrich et al. 2010) via activation of the renin-angiotensin system and thus promotion of vasoconstriction, hypertension and ultimately arterial stiffening (Ferrario and Strawn 2006).

A strategy with therapeutic potential to influence immune function, inflammation and MP levels is regular moderate intensity aerobic exercise, which can enhance immune function and reduce systemic inflammation in both the general population (Gleeson et al. 2011; Nieman and Pedersen 1999) and in other chronically inflamed patient groups (e.g. cardiovascular disease, chronic obstructive pulmonary disease) (Fernandes et al. 2018; Goldhammer et al. 2005).

In the RTR population, aerobic exercise interventions have been shown to improve endothelial-dependent flow-mediated dilation (Cosio-Lima et al. 2006), peak aerobic capacity and muscular strength (Riess et al. 2014), and quality of life (Shakoor et al. 2013), as well as cause no detriment to renal function (Mosconi et al. 2016). Previous efforts have also been made to characterise the immune and inflammatory response to aerobic exercise in the CKD and HD populations (Table 2.6 and 2.7, Chapter 2), although there is no such research to date in the RTR population. In a pre-dialysis CKD population, an acute 30 minute bout of walking exercise had no effect on T lymphocyte or monocyte activation, but increased neutrophil responsiveness to a bacterial challenge and created an anti-inflammatory environment via increased IL-10 circulating concentration (Viana et al. 2014). However, RTRs represent a unique population due to their immunosuppressive regimen, and as such these results cannot be transferred. Van Craenenbroeck et al demonstrated that, following a maximal intensity cardiopulmonary exercise test, CKD patients displayed an increased proportion of circulating pro-inflammatory $CD16^{+}$ monocytes, whilst IL-6 and MCP-1 were unchanged (Van Craenenbroeck et al. 2014). However, maximal-intensity aerobic exercise can suppress immune function (Gleeson 2007) and is not a form of exercise that untrained RTRs could reasonably be expected to complete regularly given their reduced physical capacity. Similarly, in healthy individuals, high-intensity exercise can alter the distribution of lymphocyte and monocyte subsets and their function (Nieman and Pedersen 1999; Simpson et al. 2007) as well as the systemic pro-/anti-inflammatory cytokine balance (Peake et al. 2005). However, these effects may arguably be expected to occur at a lower intensity in RTRs due to their suppressed immune systems and generally lower physical activity and cardiorespiratory fitness levels (Painter et al. 2002; Zelle et al. 2011). Additionally, aerobic exercise training can both reduce circulating MP levels and decrease vascular resistance possibly via reduced ACE-mediated renin-angiotensin system activation in the general population (Babbitt et al. 2013; Fagard 2006; Wahl et al. 2014) however this is yet to be investigated in the RTR population. Finally, very little is known about how exercise influences chemokine concentrations in RTRs – an aerobic exercise-induced reduction, as has been seen in IL-8 and MCP-1 in metabolic syndrome patients (Trøseid et al. 2004), would be expected to hinder atherosclerosis progression and thus minimise CV risk. As such, there is a need to

investigate the influence of moderate intensity aerobic exercise on all of the parameters mentioned above in RTRs.

Therefore, the aim of this study was to investigate how a short bout of moderate intensity walking exercise affects immune cell subsets, circulating pro- and anti-inflammatory cytokine levels, circulating chemokine levels and circulating MP phenotype numbers and characteristics in RTRs, in comparison to a uraemic control condition (i.e. non-dialysis dependent CKD patients) and a healthy control (HC) population. This will allow the investigation of uraemia alone (CKD group) versus uraemia combined with immunosuppression (RTR group) and a comparison with the general population (HC group). It is hypothesised that the exercise will positively impact the immune system, create an overall anti-inflammatory environment and positively impact circulating MP levels in all three groups.

7.3 Methods

Ethics

All aspects of the study were given a favourable ethical opinion by the NHS East Midlands Research Ethical Committee (ref.15/EM/0391) and the University Hospitals of Leicester Ethical Committee (UHL 11444). The trial was retrospectively registered with the International Standard Randomised Controlled Trials Registry (ISRCTN38935454).

Recruitment

Patients were deemed fit to participate by a consultant nephrologist prior to being approached during their routine clinic appointments. After discussing the study with the researcher and having at least 48 hours to consider their participation, they gave written informed consent. HC participants were recruited from the local community and University staff. Patients and HCs were made aware of their ability to withdraw from the study at any time without the need to provide a reason.

Exclusion Criteria

The general exclusion criteria for all participants were:

1. Under 18 years old
2. Currently dieting or exhibiting extreme dietary habits
3. Recently had an infection (last 6 weeks)
4. Currently pregnant
5. Physically incapable of walking for 20 minutes at a brisk pace, to be mutually decided upon by the participant and the investigators
6. Have insufficient command of English to understand the patient information sheet and give informed consent
7. Inability to give informed consent for any reason

The patient-specific exclusion criteria were:

1. Age < 18 years
2. Received kidney transplant less than 6 months prior to study entry
3. Any element of study assessment protocol considered by own clinician to be contraindicated due to physical impairment, co-morbidity or any other reason

Whilst the HC exclusion criteria were:

1. Evidence of kidney disease (e.g. urinary problems)
2. Personal history of cardiovascular disease, metabolic disease, high blood pressure or dyslipidaemia (abnormal blood fat (triglyceride) or cholesterol)
3. Diabetes
4. Currently taking drugs known to affect digestion, metabolism or inflammation, medical or illegal (e.g. anabolic steroids, marijuana, amphetamines, thyroid prescription drugs, corticosteroids).

Study Design

For a full description of the protocol implemented in this study, see the previously published protocol paper (Highton et al. 2017). This study was completed over two laboratory visits completed within 2 weeks for each participant. Participants arrived to both visits non-fasted (as the ethical review process deemed a fasted exercise study to be unsuitable for the patient groups) but having refrained from caffeine, alcohol and exercise for the previous 24 hours.

Visit 1

On the first visit, the following assessments were completed: basic anthropometry (height, weight, hip and waist circumference), body composition analysis (InBody 370, California, USA), Non-Invasive Cardiac Output Monitoring (Starling SV NICOM – Cheetah Medical, Maidenhead, UK – provides basic cardiac function measures, e.g. stroke volume, total peripheral resistance (Squara et al. 2007) and habitual physical activity assessed by the Leisure Time Exercise Questionnaire (LTEQ – this assesses the amount of time spent doing physical activity of various intensities per week, gives each category of intensity a

certain weighting and then creates a total leisure time activity score (Godin and Shephard 1985) – a copy of this questionnaire is included in Appendix 6). Following this, the Incremental Shuttle Walk Test (ISWT) was completed as described in Chapter 3. Subsequently, the Endurance Shuttle Walk Test (ESWT) was completed, again as described in Chapter 3. Using a conversion chart, 85% of the final speed reached in the ISWT was applied – the patient was then required to maintain this pace for 20 continuous minutes up and down the 10-metre course in the ESWT. The purpose of these tests was to determine the required pace for the ESWT and to act as a familiarisation for visit 2. Patients who were not able to complete the 20-minute ESWT were withdrawn from the study and did not attend visit 2. These exercise tests have been validated in a Chronic Obstructive Pulmonary Disease patient population (Revill et al. 1999; Singh et al. 1992) and have been previously used in CKD patients (Greenwood et al. 2012).

Visit 2

Participants arrived at the laboratory in the morning (8-9am). They then completed the ESWT at the same pace as completed on visit 1 for 20 minutes, with venous blood samples taken pre-exercise, immediately post-exercise and 1-hour post-exercise (Figure 7.1).

Figure 7.1. Schematic representation of the study protocol for visit 2.
↓ = Venous blood sample collection

Venous blood sampling and storage

The venous blood sample collection, processing and storing protocol is described in detail in Chapter 3. Briefly, venous blood was drawn from the antecubital vein using a 21-guage needle into pre-treated monovettes. Blood was drawn into sodium-heparin-treated (16 IU/mL blood) monovettes for same-day flow cytometry analysis of immune cells, K_3EDTA-treated (1.6mg/mL blood) monovettes for same-day full cell count analysis and future cytokine and chemokine analysis, sodium citrate-treated (0.106 mol/L)

monovettes for future MP analysis and z-gel (serum)-treated monovettes (no anticoagulant) for renal profile analysis.

Sample preparation procedures are described in Chapter 3. Briefly, K_3EDTA-treated venous blood was centrifuged (10 mins, 2,500g, 4°C) and sodium citrate-treated venous blood was double-centrifuged (15 mins, 2,500g, 20°C, supernatant aliquoted and centrifugation repeated) for future cytokine/chemokine and MP analysis, respectively. Samples were stored at -80°C for future batch analysis. Z-gel (serum) monovettes were sent to the pathology laboratory at the Leicester Royal Infirmary for full renal profile analysis.

Full Blood Count

Full blood cell counts from each timepoint were obtained by analysing venous whole blood collected into EDTA-treated monovettes using an automatic haematology analyser (Yumizen, Horiba, Northampton, UK) on the day of collection. This quantified neutrophils at each timepoint, as these were not included in the immune cell flow cytometry panel (below). Plasma volume changes were calculated using haemoglobin and haematocrit values obtained from the haematology analyser and previously defined equations (Dill and Costill 1974). No significant changes were observed in plasma volume ($p \geq .761$) and as such related results (i.e. immune cells, cytokines, chemokines, MPs) were not adjusted.

Immune Cell Phenotyping

Monocyte subsets were categorised as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (Ziegler-heitbrock, Thomas, and Hofer 2013). Monocyte ACE expression was assessed using CD143, with IgG1 as a negative control. T cells were categorised as helper ($CD3^{+}CD4^{+}$) cytotoxic ($CD3^{+}CD8^{+}$) and regulatory ($CD4^{+}CD25^{+}CD127^{-}$) T cells. B Cells were identified as $CD3^{+}CD19^{+}$. Whole blood was incubated with fluorochrome-conjugated antibodies in the dark at room temperature for 15 minutes. The antibodies used were anti-CD3 FITC, anti-CD4 PE, anti-CD8 PECy5, anti-CD14 FITC, anti-CD16 PE, anti-CD19 APC, anti-CD56 PECy7, anti-143 APC, anti-IgG1 APC, and a T-reg cocktail containing anti-CD4 FITC, anti-CD25 PECy7 and anti-CD127 Alexafluor 647. All antibodies were purchased from BD Biosciences (Oxford, UK) apart from anti-CD143, which was purchased from Biolegend (San Diego, California).

Red blood cells were lysed using BD lysing solution (BD Biosciences) for 10 minutes in the dark at room temperature, after which the samples were twice washed using phosphate-buffered saline (PBS) and centrifugation at 400g for 6 minutes – the supernatant was discarded after each wash. The samples were then re-suspended in PBS and analysed on a BD FACScalibur (BD Biosciences) flow cytometer, with all analyses collecting 100,000 total events on a standard forward and side scatter plot.

Analysis and enumeration of immune cell subsets was completed using BD CellQuest software (version 5.4) by a single operator. Neutrophils were first removed from the analyses using a side-scatter versus CD16 plot (Figure 7.2). Immune cells were then gated on a standard forward/side scatter profile, after which their subsets were gated using expression of the markers mentioned above. Figure 7.3 shows examples of the gating strategy used. Absolute counts of leukocyte subsets were calculated by multiplying their percentages obtained using flow cytometry with the relevant leukocyte counts obtained using the haematology analyser.

Microparticle Analysis

The protocol used to analyse MPs is described in detail in Chapter 3. Briefly, MPs were labelled using specific antibodies conjugated to fluorochromes, and enumerated via flow cytometry (BD Accuri C6). MPs were characterised based on size, Annexin-V expression as a marker of phosphatidylserine externalisation, TF (CD142) expression and their cellular origin, i.e. platelet ($CD42b^+$), neutrophil ($CD66b^+$), monocyte ($CD14^+$) or endothelial cell ($CD144^+$).

Cytokine and Chemokine Analysis

Plasma concentrations of circulating cytokines (IL-2, IL-6, IL-10, IL-17a, TNF-α) and chemokines (IL-8, RANTES, MIG, MCP-1, IP-10) were multiplexed and analysed using a cytometric bead array technique (BD Biosciences, Oxford, UK) in combination with a BD Accuri C6 Flow Cytometer, as described previously (Chapter 3). As mentioned in previous chapters, any cytokine value that fell below the minimum detectable threshold (0.274 pg/mL) were omitted from subsequent statistical analysis and are not included in the results presentation. The number of included samples in each outcome measure is included within the presentation of that variable.

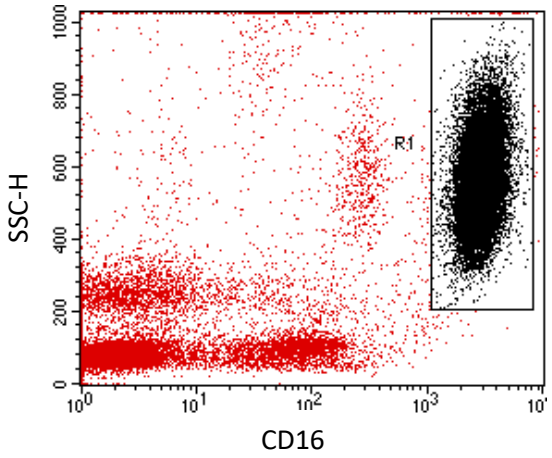

Figure 7.2. Demonstration of the gating strategy used to remove neutrophils from the panel using a side-scatter vs CD16 graph.

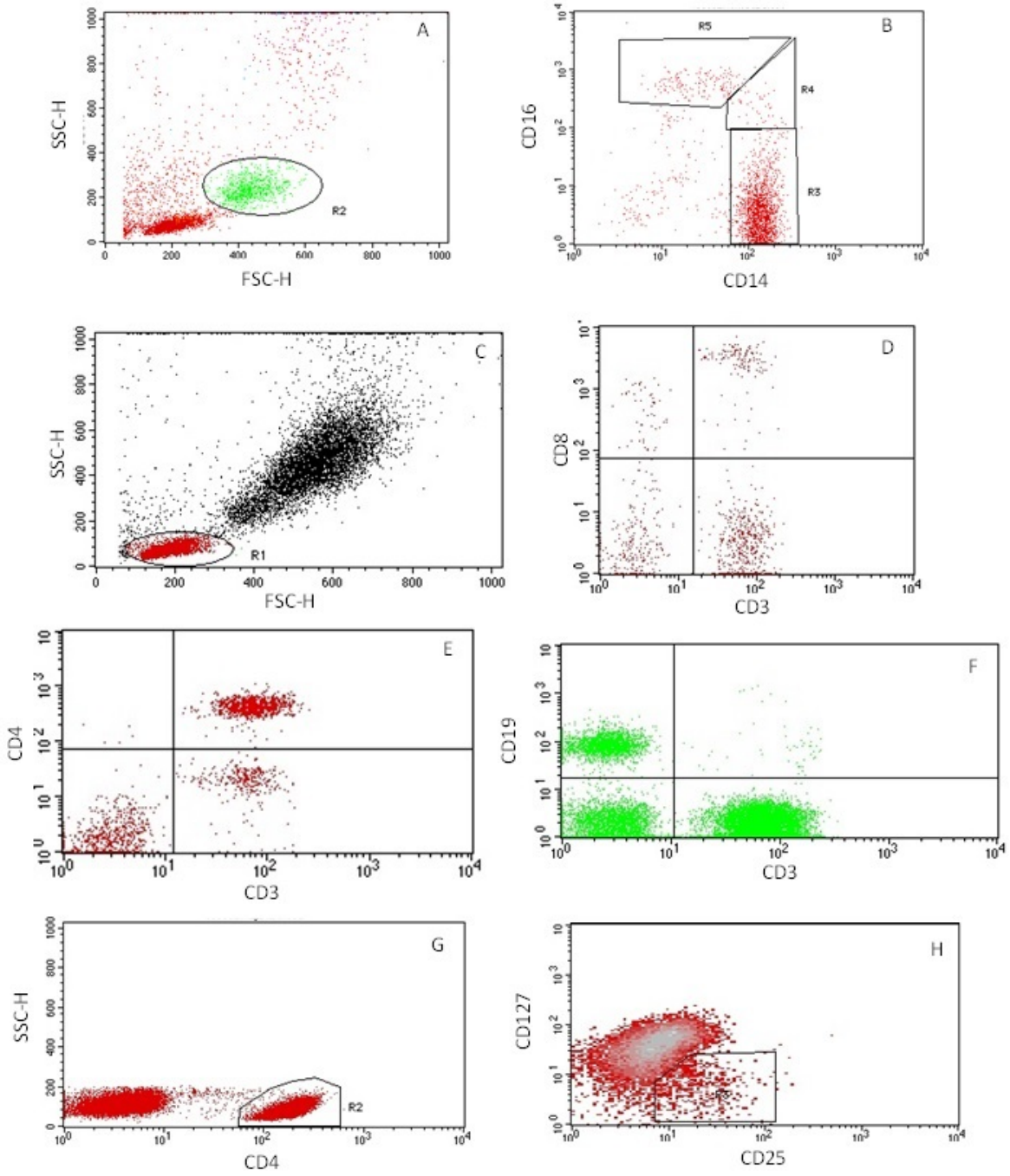

Figure 7.3. Demonstration of the gating strategy used to identify immune cell subsets. A: Total monocyte gating. B: Monocyte subsets (R3 = classical ($CD14^{++}CD16^{-}$), R4 = intermediate ($CD14^{++}CD16^{+}$), R5 = non-classical ($CD14^{+}CD16^{++}$). C: Total lymphocyte gating. D: Gating $CD8^{+}$ cytotoxic T lymphocytes in the upper right quadrant. E: Gating $CD4^{+}$ helper T lymphocytes in the upper right quadrant. F: Gating B lymphocytes ($CD3^{-}CD19^{+}$) in the upper left quadrant. G: Initial T-reg gating, identifying $CD4^{+}$ lymphocytes. H: Secondary T-reg gating, back-gated onto plot G, further identifying the $CD4^{+}$ lymphocytes that are $CD25^{+}CD127^{-}$. Not all graphs display 100% of acquired cells – this has been altered independently to allow ease of gating.

Statistical Analysis

Any non-normally distributed data was first logarithmically transformed to allow the use of parametric testing. All data was either normally distributed or successfully logarithmically transformed – if data required transformation prior to analysis this is listed when that variable is presented. As this study consisted of three different populations and therefore was unrandomised, baseline demographic and clinical results were compared at baseline using a one-way ANOVA, with Bonferroni correction to elucidate significant results in post-hoc testing. Baseline differences in immune cell subsets, cytokines, chemokines and MPs were assessed using a one-way ANOVA, (other than the MP data which was assessed using independent samples t-tests as only the HC and RTR conditions were analysed) whilst the impact of the walking bout was assessed using a mixed-design ANOVA, allowing the detection of a time*group interaction effect. When Mauchly's Test of Sphericity was violated, the Greenhouse-Geisser p value was reported. If a significant time*group interaction effect was detected, post-hoc testing was used to analyse multiple comparisons and the derived p-values were adjusted using the Bonferroni method (McHugh 2011). Partial eta squared (η^2) was used to estimate effect sizes for all ANOVAs. Effect sizes were classified as small (0.2), medium (0.5) and large (0.8) (Cohen 1988). For all analyses, $p < .05$ was considered statistically significant. All descriptive data are presented as 'mean ± standard deviation' whilst all outcome data is reported as 'mean ± standard error of the mean' when presented in a figure and 'mean ± SD' when presented in a table or discussed in the text. All statistical analysis was completed on IBM SPSS version 23.0 (Chicago, Illinois) and all graphs were produced on GraphPad Prism (v.6 GraphPad Software Inc., CA, USA).

7.4 Results

Participants

A sample size calculation completed using data gained from the most relevant previous research (Viana et al. 2014) (total leukocyte count, pre to 1-hour post 30 mins of moderate intensity walking exercise, p = .01, ES = .27) determined a sample size of n = 45 would be sufficient to detect significant leukocyte changes in this study. Following participant recruitment, a total of 47 volunteers participated in the study, comprised of 16 HC participants, 15 RTRs and 16 non-dialysis dependent CKD patients. Their anthropometric, body composition, demographic, body composition, primary diagnosis, comorbidity and clinical laboratory information is presented in Table 7.1.

The patients and healthy participants were well matched for basic demographic and body composition results. Aside from hypertension, which was significantly more prevalent in both the patient groups than the healthy participants, the presence of other comorbidities was not significantly different between groups.

Both patient groups also displayed clinical laboratory results that typically characterise renal insufficiency, namely significantly reduced eGFR and significantly increased circulating concentrations of urea and creatinine.

Cardiovascular and physical activity results

All results obtained by the NICOM technique and the LTEQ are included in Table 7.2. There were no significant differences in resting cardiac output, stroke volume, total peripheral resistance or the weekly time spent in leisure activities between the groups.

Physical performance

All performance data obtained from the completion of the ISWT and the ESWT is presented in Table 7.3. HC participants walked significantly further than both patient groups in the ISWT and had a significantly lower RPE in the ESWT. No other significant differences were seen between groups.

	HC (n = 16)	RTR (n = 15)	CKD (n = 16)	P Value
Age (years)	52.2 ± 16.2	52.8 ± 14.5	54.8 ± 16.3	.894
Male gender, n (%)	9 (56.3)	13 (86.7)	8 (50.0)	.079
Height (m)	1.71 ± 0.11	1.70 ± 0.08	1.67 ± 0.13	.516
Weight (kg)	73.51 ± 13.50	73.14 ± 14.05	78.53 ± 17.55	.541
BMI (kg/m^2)	25.55 ± 3.04	25.25 ± 3.97	28.33 ± 6.19	.129
Skeletal Muscle Mass (kg)	26.49 ± 7.53	29.06 ± 7.91	29.41 ± 6.73	.485
Body Fat %	27.84 ± 8.12	27.95 ± 11.62	31.69 ± 11.76	.514
Ethnicity, n (%)				
Caucasian	14 (87.50)	10 (66.67)	15 (93.75)	.177
Asian	1 (6.25)	4 (26.67)	1 (6.25)	.155
Black	1 (6.25)	1 (6.67)	0 (0)	.599
Primary Diagnosis, n (%)				
Diabetic Nephropathy		0 (0)	0 (0)	
Hypertensive Nephropathy		1 (6.7)	0 (0)	
Glomerulonephritis		2 (13.3)	1 (6.3)	
Pyelonephritis		3 (20.0)	2 (12.5)	
Polycystic Kidney Disease	-	4 (26.7)	1 (6.3)	-
IgA Nephropathy		1 (6.7)	9 (56.3)	
Other		2 (13.3)	2 (12.5)	
Unknown		2 (13.3)	1 (6.3)	
Current Transplant Duration (years)		6.25 ± 5.63	-	
Comorbidities, n (%)				
Diabetes	0 (0)	2 (13.3)	4 (25.0)	.109
Hypertension	0 (0)	11 (73.3)	8 (50.0)	<.001*
Ischaemic Heart Disease	0 (0)	1 (6.7)	2 (12.5)	.367
Previous Stroke	0 (0)	1 (6.7)	0 (0)	.352
Dyslipidaemia	0 (0)	1 (6.7)	2 (12.5)	.791
Previous/Current Smoker	4 (25.0)	0 (0)	4 (25.0)	.108
Clinical Laboratory/Full Blood Count				
eGFR (mL/min/1.73m^2)	85.58 ± 6.10	51.73 ± 19.77	61.94 ± 21.03	<.001**
Urea (mmol/L)	5.76 ± 1.44	10.17 ± 5.88	8.36 ± 3.16	.027*
Creatinine (µmol/L)	78.67 ± 14.88	144.33 ± 49.21	110.63 ± 47.14	.001*
Haemoglobin (g/L)	143.67 ± 29.94	138.91 ± 13.76	146.13 ± 22.12	.541
Haematocrit (L/L)	0.42 ± 0.09	0.40 ± 0.05	0.44 ± 0.07	.285
Platelets (x10^9/L)	148.67 ± 103.51	55.18 ± 43.55	237.00 ± 94.79	<.001 #+
White Cell Count (x10^9/L)	5.31 ± 1.57	6.51 ± 2.39	6.28 ± 1.50	.184
Neutrophils (x10^9/L)	2.67 ± 0.83	3.81 ± 1.67	3.82 ± 1.28	0.27*
Lymphocytes (x10^9/L)	1.89 ± 0.71	1.92 ± 0.87	1.70 ± 0.61	.689
Monocytes (x10^9/L)	0.42 ± 0.15	0.42 ± 0.34	0.50 ± 0.16	.464

Table 7.1. Participant demographic, diagnostic and clinical laboratory results.

* = significantly lower in HC condition compared to both RTR and CKD.

** = significantly higher in HC condition compared to both RTR and CKD.

= significantly lower in RTR condition compared to both HC and CKD.

+ = significantly higher in CKD than RTR and HC.

P values obtained from one-way ANOVA. All baseline demographic and clinical information was normally distributed. Data is presented as 'mean ± SD'.

	HC (n = 16)	RTR (n = 15)	CKD (n = 16)	P Value
Cardiac Output (L/min)	6.85 ± 2.05	7.31 ± 1.59	6.76 ± 1.77	.680
Stroke Volume (ml/beat)	108.98 ± 25.75	107.77 ± 24.49	104.84 ± 22.96	.891
Total Peripheral Resistance (dynes/sec/cm^5)	1173.1 ± 333.8	1167.2 ± 273.1	1283.1 ± 344.9	.536
LTEQ Score (AU)	38 ±21	33 ±16	40 ± 26	.633

Table 7.2. Cardiovascular results obtained by NICOM and the leisure time score obtained using the LTEQ. P values obtained from one-way ANOVA. This data was normally distributed. Data is presented as 'mean ± SD'.

	HC (n = 16)	RTR (n = 15)	CKD (n = 16)	P Value
ISWT distance (m)	729 ± 178	547 ± 164	518 ± 181	.003**
ISWT HR (bpm)	119 ± 24	111 ± 17	115 ± 33	.679
ISWT RPE	13 ± 2	14 ± 2	13 ± 2	.512
ESWT HR (bpm)	113 ± 19	109 ± 18	119 ± 25	.485
ESWT RPE	12 ± 2	15 ± 3	13 ± 2	.024*

Table 7.3. ISWT and ESWT performance results.
* = significantly lower in HC condition compared to both RTR and CKD.
** = significantly higher in HC condition compared to both RTR and CKD.
P values obtained from one-way ANOVA. This data was normally distributed. Data is presented as 'mean ± SD'.

Immune cell phenotypes

All immune cell phenotype data is presented in Table 7.4.

Absolute neutrophil numbers, as obtained from the haematology analyser, displayed significant between-group differences at baseline ($p = .027$), with the HC condition displaying reduced circulating neutrophil numbers when compared to the CKD patients only ($p = .046$). A significant effect of time was revealed ($p < .001$, $\eta^2 = .224$), with post-hoc analysis revealing that there was a significant increase from baseline to 1-hour post-exercise ($p = .001$) and from immediately post to 1-hour post-exercise ($p = .017$) in all groups. However, there was no time*group interaction effect ($p = .085$, $\eta^2 = .103$), suggesting that the intervention affected all groups similarly. These relationships are displayed in Figure 7.4. Absolute monocyte numbers showed no group differences at baseline ($p = .447$) and no time*group interaction effect ($p = .821$, $\eta^2 = .017$) (post-hoc analysis revealed a total sample size of n = 183 would have been

necessary in order to observe a significant interaction effect). However, significant changes were observed in the relative monocyte subset composition. The percentage of classical monocytes showed a significant difference at baseline ($p = .011$), with the RTRs displaying a greater percentage than the HCs only ($p = .011$). This percentage also significantly increased in all groups throughout the trial ($p = .001$, $\eta^2 = .162$). Post-hoc analysis revealed a significant increase from baseline to 1-hour post-exercise ($p = .008$). However, there was no time*group interaction effect ($p = .985$, $\eta^2 = .004$), suggesting that the influence of the exercise did not differ between groups.

Baseline differences were also observed in the percentage of intermediate monocytes within the monocyte population ($p < .001$), with the CKD patient displaying elevated levels compared to both the HCs ($p < .001$) and the RTRs ($p = .009$). There was also a significant effect of time ($p = .002$, $\eta^2 = .131$), elucidated as a decrease from baseline to 1-hour post-exercise ($p < .001$) in all groups. However, there was no time*group interaction effect ($p = .088$, $\eta^2 = .093$).

No baseline differences were observed in the percent of non-classical monocytes within the total monocyte population ($p = .076$). However, a significant effect of time was demonstrated for the percentage of non-classical monocytes within the monocyte population ($p < .001$, $\eta^2 = .197$). There was an initial increase from baseline to immediately after exercise in all groups ($p < .001$), followed by a decrease at 1-hour post-exercise ($p < .001$), though there was no time*group interaction effect ($p = .065$, $\eta^2 = .099$), suggesting the response did not differ between groups. The relative monocyte subset composition is displayed in Figure 7.5.

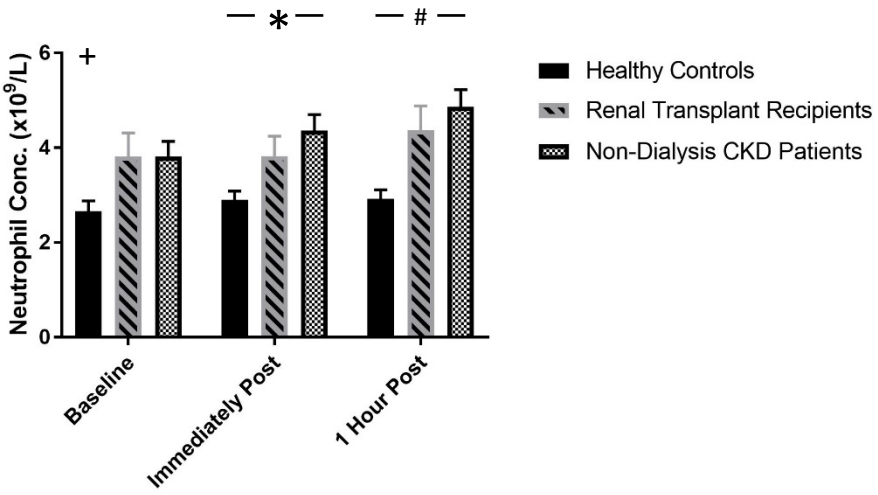

Figure 7.4. Circulating neutrophil concentrations. Data are presented as 'mean ± SEM'. * = significantly different from baseline. # = significantly different from immediately post-exercise. + = significantly different from the CKD population. This data was normally distributed. (HC n = 16, RTR n = 15, CKD n = 16).

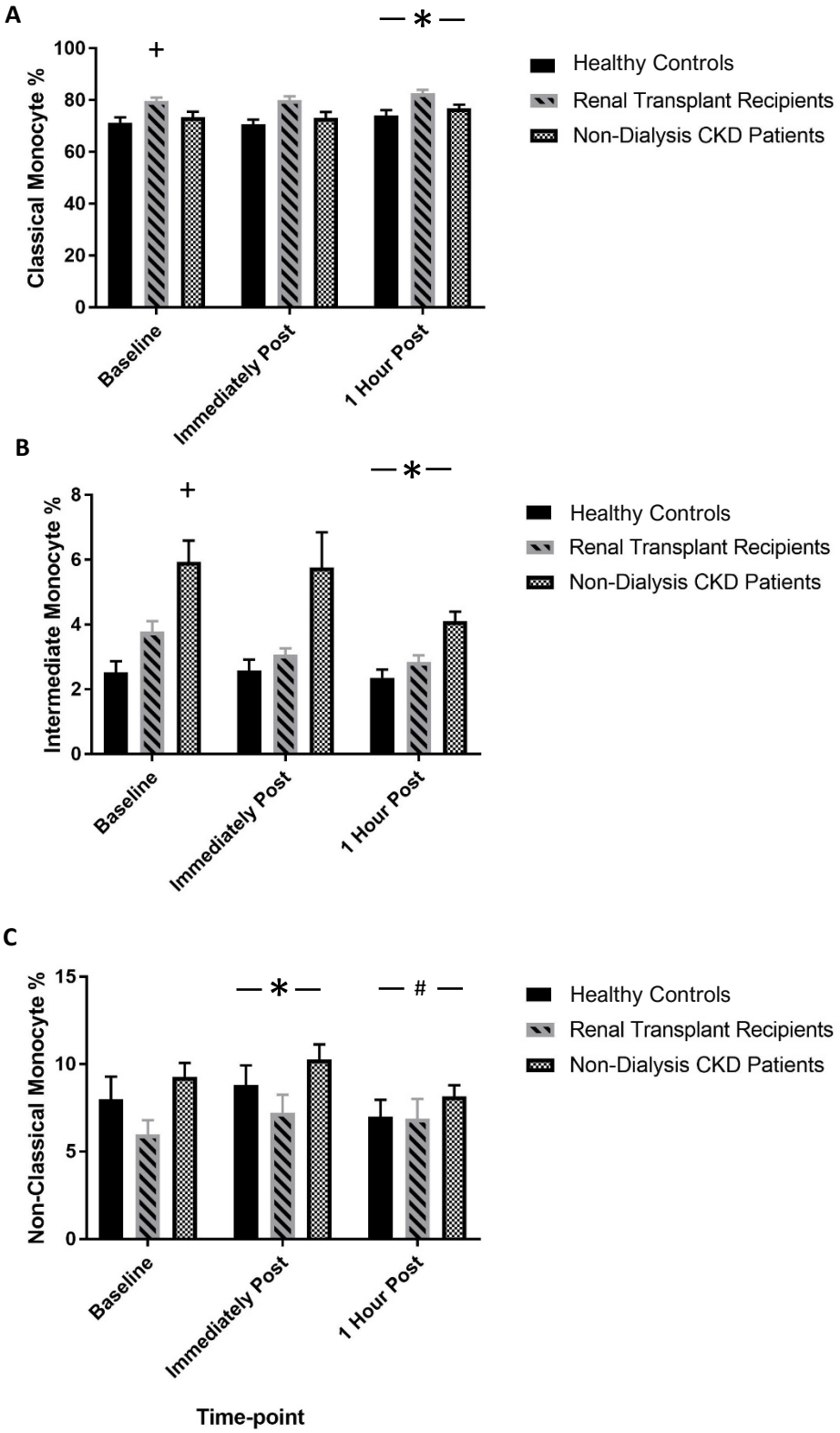

Figure 7.5. The relative distribution of the three monocyte subsets: classical (A); intermediate (B); and non-classical (C). Data is presented as 'mean ± SEM'. * = significantly different from baseline. # = significantly different from immediately post-exercise. + = significantly different from the other two populations (only compared at baseline). This data was normally distributed (HC n = 16, RTR n = 15, CKD n = 16).

	HC (n = 16)			RTR (n = 15)			NDD (n = 16)		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Neutrophil conc. (/µl)	267 ± 83	291 ± 70	292 ± 76	381 ± 167	382 ± 141	438 ± 170	382 ± 128	437 ± 134	487 ± 144
Monocyte conc. (/µl)	404 ± 152	430 ± 137	410 ± 97	507 ± 396	507 ± 384	560 ± 424	504 ± 156	553 ± 177	530 ± 186
% Classical	71.25 ± 8.84	70.81 ± 7.00	74.04 ± 8.55	79.79 ± 4.73	80.14 ± 5.42	82.68 ± 5.43	73.47 ± 8.73	73.27 ± 8.80	76.87 ± 5.74
% Intermediate	2.52 ± 1.42	2.58 ± 1.38	2.36 ± 1.04	3.80 ± 1.20	3.07 ± 0.79	2.85 ± 0.82	5.94 ± 2.65	5.77 ± 4.31	4.11 ± 1.16
% Non-classical	8.00 ± 5.14	8.83 ± 4.48	7.02 ± 3.76	5.99 ± 3.16	7.25 ± 3.96	6.90 ± 4.34	9.29 ± 3.15	10.29 ± 3.40	8.17 ± 2.54
Classical conc. (/µl)	291 ± 131	309 ± 121	303 ± 84	410 ± 335	416 ± 333	470 ± 366	371 ± 129	404 ± 157	440 ± 153
Intermediate conc. (/µl)	9 ± 4	11 ± 6	9 ± 4	19 ± 15	16 ± 12	20 ± 11	31 ± 20	33 ± 30	20 ± 9
Non-classical conc. (/µl)	30 ± 19	37 ± 22	29 ± 18	31 ± 32	33 ± 27	40 ± 31	46 ± 22	56 ± 25	50 ± 20
% ACE+ classical	3.11 ± 7.6	3.14 ± 6.86	2.94 ± 6.73	8.58 ± 7.60	8.64 ± 7.93	10.21 ± 8.58	1.71 ± 2.42	1.03 ± 1.12	2.84 ± 4.70
% ACE+ intermediate	8.91 ± 8.84	9.39 ± 8.52	7.00 ± 7.12	29.49 ± 17.10	33.91 ± 19.35	38.40 ± 21.69	8.12 ± 9.56	5.14 ± 3.97	4.46 ± 4.38
% ACE+ non-classical	0.81 ± 1.65	0.57 ± 0.76	0.66 ± 0.84	7.26 ± 6.47	7.92 ± 4.39	8.40 ± 6.25	2.72 ± 4.60	2.33 ± 5.91	1.23 ± 1.39
Classical ACE GMFI	174.8 ± 84.1	185.8 ± 117.7	186.4 ± 124.4	40.1 ± 8.76	39.4 ± 7.86	42.3 ± 9.03	111.5 ± 43.9	113.4 ± 74.4	94.8 ± 32.1
Intermediate ACE GMFI	385.2 ± 489.3	260.3 ± 128.8	268.0 ± 199.3	68.1 ± 31.7	60.1 ± 15.59	65.2 ± 22.60	270.2 ± 152.2	254.4 ± 125.3	247.4 ± 99.1
Non-classical ACE GMFI	121.8 ± 38.7	113.3 ± 29.0	126.9 ± 68.5	58.6 ± 23.2	81.1 ± 130.8	58.8 ± 31.7	178.2 ± 100.3	134.5 ± 67.1	145.9 ± 71.3
Lymphocyte conc. (/µl)	1850 ± 7.5	1930 ± 773	1720 ± 652	1930 ± 774	1780 ± 687	1640 ± 568	1700 ± 608	1820 ± 646	1710 ± 557
% T-reg	8.14 ± 2.17	9.72 ± 2.91	9.55 ± 2.73	5.59 ± 2.08	6.29 ± 3.15	6.55 ± 2.83	6.92 ± 1.41	6.66 ± 1.30	7.19 ± 1.67

T-reg conc. (/µl)	147 ± 51	180 ± 57	155 ± 44	101 ± 42	106 ± 52	100 ± 46	116 ± 41	119 ± 43	120 ± 45
% CD4+	34.36 ± 10.27	34.98 ± 9.37	36.18 ± 11.71	35.43 ± 12.02	33.37 ± 10.38	34.89 ± 10.70	42.25 ± 7.94	41.29 ± 9.44	45.05 ± 9.43
% CD8+	19.55 ± 5.62	20.22 ± 7.54	19.12 ± 7.14	29.95 ± 15.52	31.63 ± 15.77	29.84 ± 16.18	25.32 ± 10.32	25.53 ± 10.68	25.17 ± 10.09
CD4+ conc. (/µl)	655 ± 407	670 ± 313	612 ± 292	661 ± 267	579 ± 221	570 ± 225	688 ± 205	707 ± 177	730 ± 185
CD8+ conc. (/µl)	352 ± 125	390 ± 229	326 ± 175	650 ± 621	601 ± 480	510 ± 430	472 ± 305	511 ± 369	470 ± 326
% B cell	12.60 ± 12.20	12.60 ± 12.8	11.40 ± 2.66	9.62 ± 7.44	8.16 ± 6.70	9.74 ± 7.32	7.59 ± 2.93	7.01 ± 2.50	8.20 ± 3.07
B cell conc. (/µl)	216 ± 162	290 ± 487	199 ± 95	168 ± 112	135 ± 112	150 ± 107	134 ± 58	132 ± 56	150 ± 70

Table 7.4. All immune cell phenotype data. Conc. = concentration. T1 = Baseline, T2 = Immediately after exercise, T3 = 1 hour after exercise. No. = absolute circulating concentration, % = relative concentration with regards to the whole phenotype (i.e. % classical = the % of classical monocytes of the total monocyte pool, % CD4+ = the % of CD4+ T cells of the total lymphocyte pool). GMFI = geometric median fluorescence intensity. In order to maintain the clarity of the table, notations of significance have not been added and instead are described in the body of the text. Data is presented as 'mean ± SD'. All immune cell and phenotype data was normally distributed and not subject to transformation prior to analysis (HC n = 16, RTR n = 15, CKD n = 16).

There were comparatively less changes seen in the absolute circulating number of each monocyte subset. The number of classical monocytes showed no baseline differences ($p = .165$) was unaffected by time ($p = .167$, $\eta^2 = .041$) and displayed no time*group interaction effect ($p = .760$, $\eta^2 = .018$) (n = 174 identified in post-hoc calculation as an adequately powered sample size). However, the number of intermediate monocytes did show a between group difference ($p = .001$) – the CKD patients demonstrated a greater circulating number than the HCs ($p < .001$). The number of intermediate monocytes was unaffected by time ($p = .255$, $\eta^2 = .059$), and showed no time*group interaction effect ($p = .255$, $\eta^2 = .059$) (n = 54 identified as sufficient in post-hoc calculations). The number of non-classical monocytes displayed no baseline between-group differences ($p = .057$), was unaffected by time ($p = .100$, $\eta^2 = .054$) and displayed no time*group interaction effect ($p = .388$, $\eta^2 = .045$) (sufficient sample size from post-hoc analysis: n = 69).

The percentage of classical monocytes that positively expressed ACE (ACE^+) also showed significant differences at baseline ($p < .001$) .Post-hoc analysis revealed that RTRs had a greater percentage of ACE^+ classical monocytes than the HCs ($p < .001$) and CKD patients ($p < .001$). However, there was no effect of time ($p = .052$, $\eta^2 = .067$) or time*group interaction effect ($p = .404$, $\eta^2 = .044$) (sufficient sample size from post-hoc analysis: n = 72). Similarly, the percentage of ACE^+ intermediate monocytes also showed between-group differences at baseline ($p < .001$). Again, this was caused by RTRs displaying greater values than the HCs ($p < .001$) and CKD patients ($p < .001$). However, there was no main effect of time ($p = .686$, $\eta^2 = .008$). However, there was a significant time*group interaction effect ($p = .002$, $\eta^2 = .176$) – this was likely caused by a relative increase in the RTR patients coinciding with a relative decrease in the HC and CKD groups, however this did not reach significance in any condition ($p \geq .080$). The percentage of ACE^+ non-classical monocytes also displayed a group difference at baseline ($p = .002$) - as with the classical and intermediate monocytes, this was due to the RTRs displaying a significantly greater percentage of ACE^+ non-classical monocytes than the HCs ($p < .001$) and CKD patients ($p < .001$). However, there was no main effect of time ($p = .963$, $\eta^2 = .001$) or time*group interaction effect ($p = .718$, $\eta^2 = .023$) (sufficient sample size from post-hoc calculation: n = 135). The percentage of ACE^+ monocytes from each of the three subsets is displayed in Figure 7.6.

The degree of expression of ACE in the ACE^{+} monocytes is represented here by the GMFI (geometric median fluorescence intensity) as this value is the most resistant to the exponential nature of fluorescence intensity. The ACE fluorescence intensity of classical monocytes displayed a significant between group differences ($p < .001$). Post-hoc analysis revealed that HCs displayed greater GMFI than both RTRs ($p < .001$) and CKD patients ($p = .002$), and CKD patients displayed greater GMFI than RTRs ($p = .010$). However, there was no effect of time ($p = .878$, $\eta^2 = .003$) or time*group interaction effect ($p = .796$, $\eta^2 = .019$) (post-hoc sufficient sample size: n = 165). Similarly, the ACE GMFI on intermediate monocytes displayed a significant baseline group difference ($p = .018$). Similar to the classical monocytes, both CKD patient and HCs displayed a greater GMFI than RTRs ($p < .001$ for both groups). Again, no significant time ($p = .357$, $\eta^2 = .021$) or time*group interaction effect was revealed ($p = .631$, $\eta^2 = .024$) (sufficient sample size from post-hoc calculation: n = 129). The ACE GMFI on non-classical monocytes displayed a similar pattern to the previous two subsets: a significant baseline difference between groups ($p < .001$) characterised both CKD patients and HCs displaying significantly greater GMFI values than the RTRs ($p < .001$ and $p = .020$, respectively). Similarly, there was no significant effect of time ($p = .628$, $\eta^2 = .010$) or no time*group interaction effect ($p = .208$, $\eta^2 = .065$) (post-hoc sufficient sample size: n = 48). The monocyte subset ACE fluorescence intensity results are displayed in Figure 7.7.

Similar to the total number of monocytes, the total number of lymphocytes displayed no group differences at baseline ($p = .667$), no main effect of time ($p = .126$, $\eta^2 = .050$) and no time*group interaction effect ($p = .529$, $\eta^2 = .032$) Post-hoc sample size calculations suggested that a sample size of n = 99 would be required to elicit a significant interaction effect. However, significant findings were observed in the relative proportion of each lymphocyte subset.

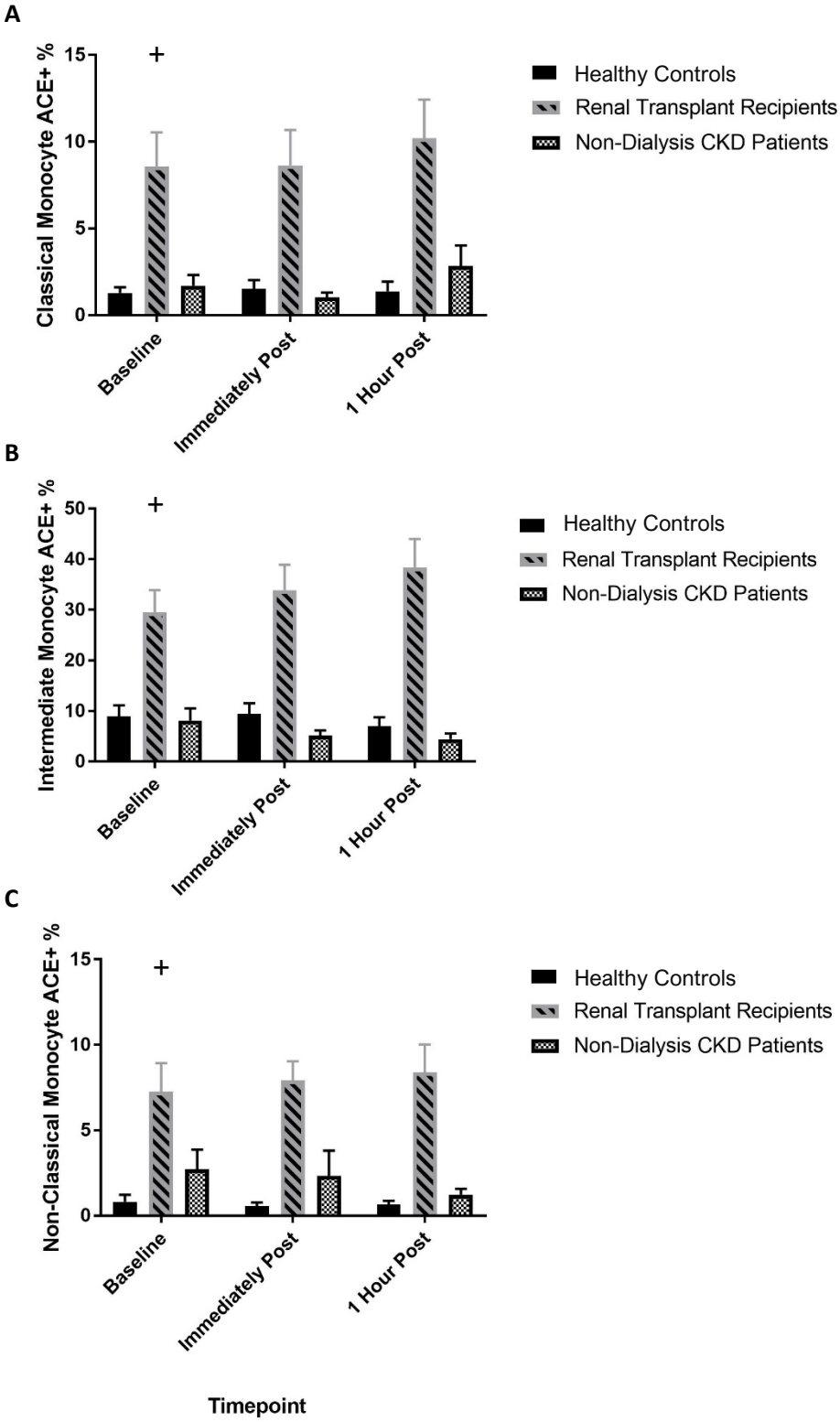

Figure 7.6. The % of each monocyte subset (classical (A), intermediate (B) and non-classical (C)) that positively expressed angiotensin-converting enzyme (ACE). Data is presented as 'mean ± SEM'. + = significantly different from the other two conditions (only compared at baseline). (HC n = 16, RTR n = 15, CKD n = 16). This data was normally distributed.

The proportion of circulating T-regs within the total lymphocyte population was significantly different between groups at baseline ($p = .002$) with the HC participants displaying a significantly greater percentage than the RTRs only ($p = .002$). There was also a significant effect of time ($p < .001$, $\eta^2 = .241$) – post-hoc analysis revealed a significant increase from baseline to immediately post-exercise ($p = .002$) and from baseline to 1-hour post-exercise ($p < .001$). Additionally, there was a significant time*group interaction effect ($p = .002$, $\eta^2 = .181$). Post-exercise, the HC population displayed a greater T-reg % than both the RTRs ($p = .002$) and the CKD patients ($p = .005$) – this relationship persisted at 1-hour post-exercise ($p = .004$ and $p = .029$, respectively). This suggests that the T-reg % increased in the HCs with respect to the CKD patients, considering the emergence of significance after the exercise bout. The absolute number of circulating T-regs also showed group differences at baseline ($p = .022$), characterised by the HCs displaying elevated numbers when compared to the RTRs only ($p = .022$). However, there was no significant effect of time ($p = .060$, $\eta^2 = .065$) or time*group interaction effect ($p = .151$, $\eta^2 = .074$). The percentage and number of circulating T-regs is displayed in Figure 7.8.

The proportion of circulating helper T cells within the total lymphocyte population showed no baseline differences ($p = .071$), was not affected by time ($p = .082$, $\eta^2 = .056$) and showed no time*group interaction effect ($p = .508$, $\eta^2 = .036$) (post-hoc sufficient sample size: n = 87). However, the percentage of cytotoxic T cells did show a significant baseline difference ($p = .042$). Post-hoc analysis revealed that the RTRs displayed a higher number of circulating cytotoxic T cells than the HCs ($p = .038$). However, there was no effect of time ($p = .054$, $\eta^2 = .066$) and no time*group interaction effect ($p = .670$, $\eta^2 = .026$) (sufficient sample size from post-hoc analysis: n = 120). As with the percentage data, the absolute number of circulating helper T cells showed no group differences at baseline ($p = .948$), was unchanged by time ($p = .592$, $\eta^2 = .010$) and showed no time*group interaction effect ($p = .335$, $\eta^2 = .050$) (post-hoc sufficient sample size: n = 63). However, contrasting the percentage data, the absolute number of circulating cytotoxic T cells showed no between-group differences at baseline ($p = .126$) and was affected by time ($p = .037$, $\eta^2 = .079$), with a significant decrease from immediately after exercise to 1-hour post-exercise ($p = .002$) in all groups. However, there was no time*group interaction effect ($p =$

.273, η^2 = .057) (post-hoc sufficient sample size: n = 57), suggesting the impact of the walking did not differ between groups.

The percentage of circulating B cells displayed no significant differences between groups at baseline (p = .388), however was significantly affected by time (p < .001, η^2 = .314), with post-hoc analysis revealing a decrease from baseline to immediately post-exercise (p = .035), but an increase from both baseline to 1-hour post-exercise (p = .010) and from immediately post to 1-hour post-exercise (p < .001). However, there was no time*group interaction effect (p = .067, η^2 = .099). The absolute number of circulating B cells also exhibited no significant differences between groups at baseline (p = .290), was not affected by time (p = .055, η^2 = .066) and showed no time*group interaction effect (p = .331, η^2 = .050) (post-hoc sufficient sample size: n = 63).

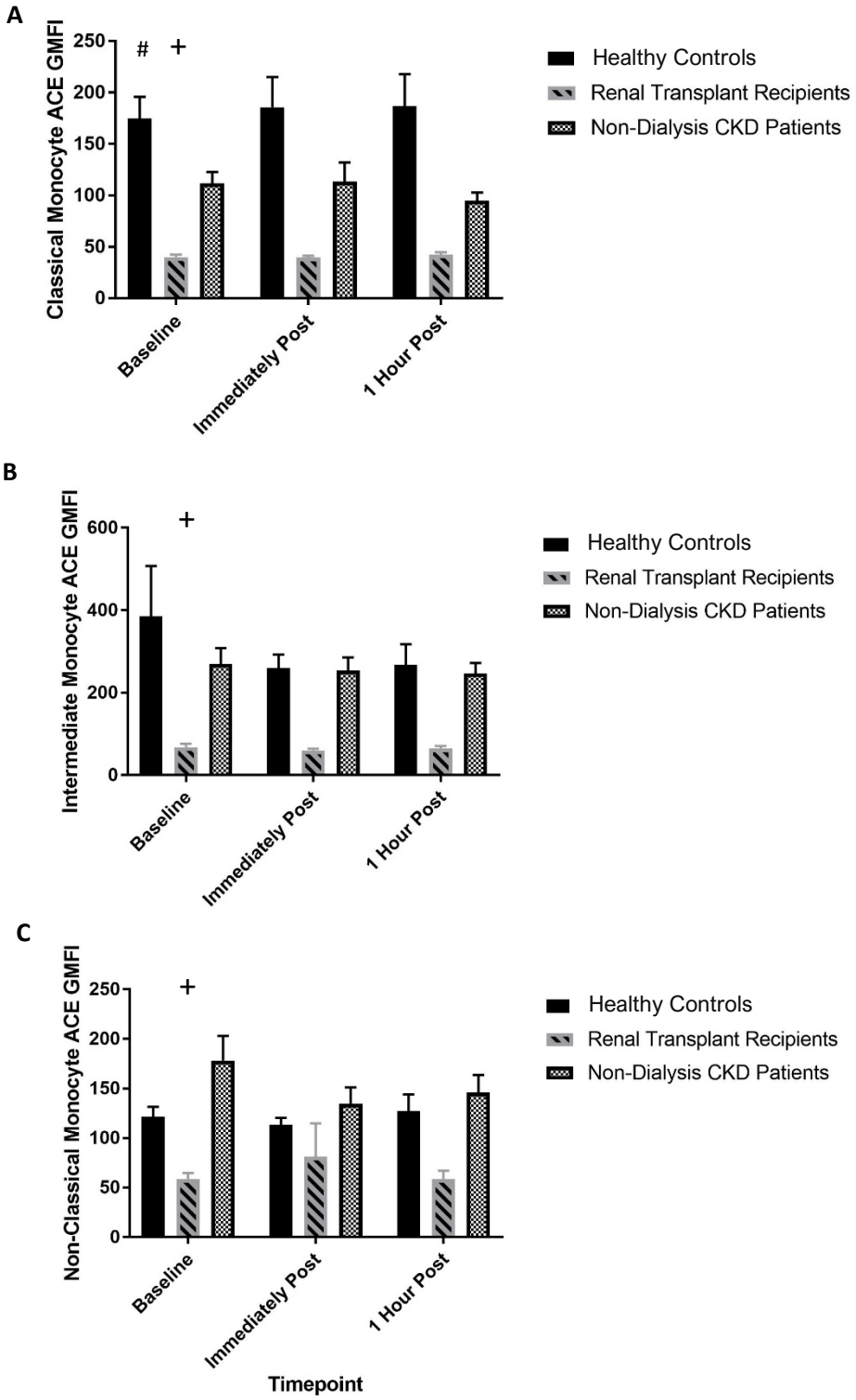

Figure 7.7. The degree of expression of ACE (represented by geometric mean fluorescence intensity – GMFI) on classical (A), intermediate (B) and non-classical (C) monocytes. Data are presented as 'mean ± SEM'. + = significantly different from the other two populations. # = significantly different from the CKD population. (HC n = 16, RTR n = 15, CKD n = 16). This data was normally distributed.

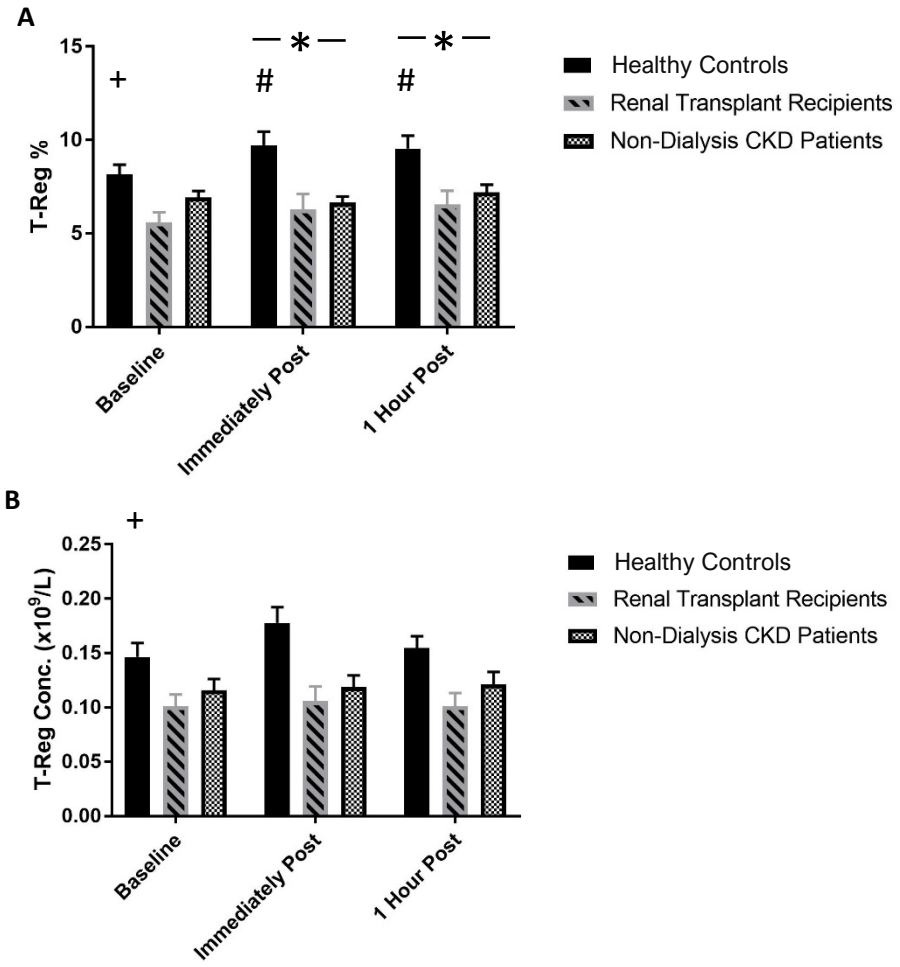

Figure 7.8. Proportion of circulating regulatory T cell percentage within the total lymphocyte population and absolute circulating concentration. Data is presented as 'mean ± SEM'. * = significantly different from baseline. + = significantly different from the RTRs. # = significantly different from the other two conditions. (HC n = 16, RTR n = 15, CKD n = 16). This data was normally distributed.

Pro- and anti-inflammatory circulating cytokines

All pro- and anti-inflammatory cytokine data is presented in Table 7.5.

All samples provide valid and detectable values for circulating IL-6. Circulating levels of IL-6 displayed significant between-group differences at baseline (p = .038), with a trend towards the HCs being lower

than the CKD patients (p = .057). However, there was no effect of time (p = .321, η^2 = .029) or time*group interaction effect (p = .443, η^2 = .046) Post-hoc sample size calculations suggested that a sample size of n = 69 would be required to elicit a significant interaction effect. Similarly, all analysed samples provided valid and detectable values for circulating IL-10 concentration. However, IL-10 showed no significant differences at baseline between groups (p = .096), were not affected by time (p = .673, η^2 = .008) but did display a significant time*group interaction effect (p = .041, η^2 = .132). Despite there being no differences at baseline, between-group differences emerged at 1-hour post-exercise, characterised by a reduction in the HCs with respect to the RTRs (p = .022). These IL-6 and IL-10 results are displayed in Figure 7.9.

Circulating IL-17a concentrations were only distinguishable above background noise in 13 patients (HC n = 3, RTR n = 6, CKD n = 4). Whilst analysis on these results was completed, as in previous chapters these results should be interpreted with caution given the small sample sizes. Circulating IL-17a concentrations displayed no between-group differences at baseline (p = .218), were unaffected by time (p = .170, η^2 = .162) and exhibited no time*group interaction effects (p = .649, η^2 = .111) (post-hoc sufficient sample size: n = 30).

Circulating TNF-α concentrations were distinguishable above background noise in 24 patients (HC n = 7, n = 9, CKD n = 9). TNF-α circulating concentrations showed no group differences at baseline (p = .220), showed no significant effect of time (p = .340, η^2 = .048) and exhibited no time*group interaction effect (p = .851, η^2 = .030) (sufficient sample size from post-hoc calculation: n = 105). This presented in Figure 7.10.

Lastly, IL-6/IL-10 ratio displayed between-group differences at baseline (p = .188), as well as no main effect of time (p = .408, η^2 = .022) and no time*group interaction effect (p = .158, η^2 = .081).

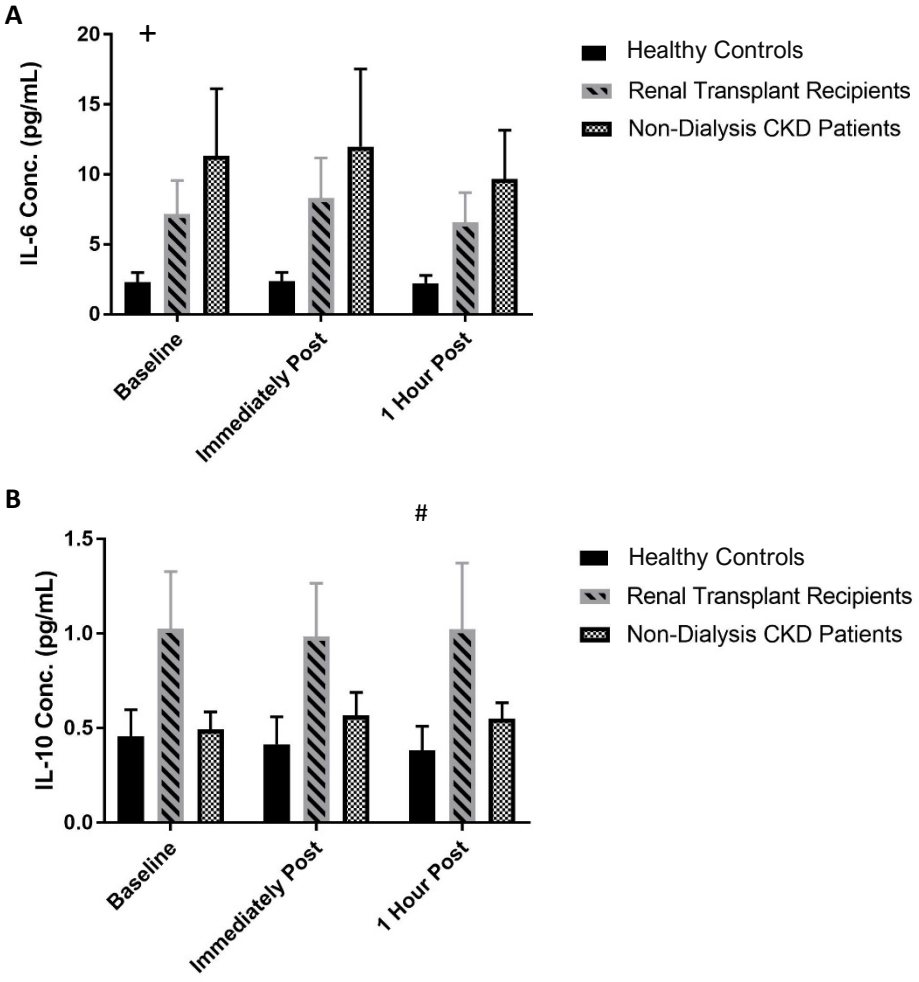

Figure 7.9. Circulation concentrations of IL-6 (A) and IL-10 (B). Data are presented as 'mean ± SEM'. + = trend towards significantly different from the other two populations (p = .057). # = significantly different from the renal transplant recipients only. (HC n = 16, RTR n = 15, CKD n = 16). This data was non-normally distributed and thus was logarithmically transformed prior to analysis.

Circulating chemokines

All circulating chemokine data is included in Table 7.6.

Circulating IL-8 levels showed no significant differences at baseline (p = .777), no main effect of time (p = .165, η^2 = .045) and no time*group interaction effect (p = .094, η^2 = .099). However, MIG showed a significant difference between groups at baseline (p < .001) – post-hoc analysis revealed that the RTRs displayed significantly elevated circulating values when compared to the HCs (p = .005) and CKD

patients ($p < .001$). A significant main effect of time was also revealed ($p = .035$, $\eta^2 = .088$), however upon post-hoc analysis there were no significant differences between time-points ($p \leq .081$).

	HC (n = 16)			RTR (n = 15)			NDD (n = 16)		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
***IL-6** (pg/mL)	2.29 ± 2.35	2.36 ± 2.21	2.22 ± 1.96	7.17 ± 9.26	8.31 ± 11.12	6.56 ± 8.40	11.33 ± 19.16	12.00 ± 22.22	9.67 ± 13.96
***IL-10** (pg/mL)	0.46 ± 0.49	0.41 ± 0.51	0.38 ± 0.44 #	1.03 ± 1.16	0.98 ± 1.09	1.02 ± 1.37	0.49 ± 0.38	0.57 ± 0.48	0.55 ± 0.33
***IL-17a** (pg/mL)	0.12 ± 0.20	0.12 ± 0.21	0.12 ± 0.23	0.92 ± 1.66	1.06 ± 1.89	0.72 ± 1.37	0.97 ± 2.27	1.45 ± 3.52	0.83 ± 1.92
***TNF-α** (pg/mL)	1.08 ± 1.67	0.98 ± 1.50	0.96 ± 1.40	3.93 ± 8.20	4.14 ± 0.86	3.08 ± 6.13	7.43 ± 15.14	7.84 ± 15.59	5.77 ± 10.45
IL-6/IL-10 Ratio	7.04 ± 5.39	9.57 ± 8.64	9.41 ± 6.97	10.61 ± 12.71	10.91 ± 8.90	9.60 ± 7.61	21.66 ± 33.49	20.79 ± 27.81	19.25 ± 26.66

Table 7.5. All pro- and anti-inflammatory cytokine data. Data are presented as 'mean ± SD'. # = significantly different from the RTR population. * indicates that data was non-normally distributed and thus logarithmically transformed prior to analysis. (IL-6 and IL-10: HC n = 16, RTR n = 15, CKD n = 16. IL-17a: HC n = 3, RTR n = 6, CKD n = 4. TNF-α: HC n = 7, RTR n = 9, CKD n = 9).

	HC (n = 16)			RTR (n = 15)			NDD (n = 16)		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
***IL-8** (pg/mL)	21.05 ± 33.04	22.34 ± 35.78	22.55 ± 36.17	38.78 ± 67.65	18.74 ± 30.94	22.33 ± 39.56	17.29 ± 16.08	18.70 ± 17.83	18.85 ± 17.96
***MIG** (pg/mL)	73.40 ± 55.00	68.95 ± 55.50	67.60 ± 55.76	199.78 ± 692.96 #	184.86 ± 715.96	174.84 ± 573.20	53.26 ± 34.43	53.56 ± 43.44	56.34 ± 50.65
MCP-1 (pg/mL)	34.90 ± 19.94	27.29 ± 12.61	25.29 ± 11.27	71.20 ± 67.34 ##	53.13 ± 55.48	54.81 ± 62.23	33.89 ± 16.32	25.37 ± 8.22	24.93 ± 6.66
IP-10 (pg/mL)	110.28 ± 33.43	115.39 ± 34.85	119.38 ± 34.28	158.38 ± 102.90	157.48 ± 83.58	150.93 ± 76.40	188.84 ± 112.82	194.40 ± 128.29	192.00 ± 140.55

Table 7.6. All circulating chemokine concentration data. All data are presented as 'mean ± SD'. # = significantly different from the other two populations, ## = significantly different from the HC population only. * indicates that data was non-normally distributed and thus logarithmically transformed prior to analysis. (HC n = 16, RTR n = 15, CKD n = 16).

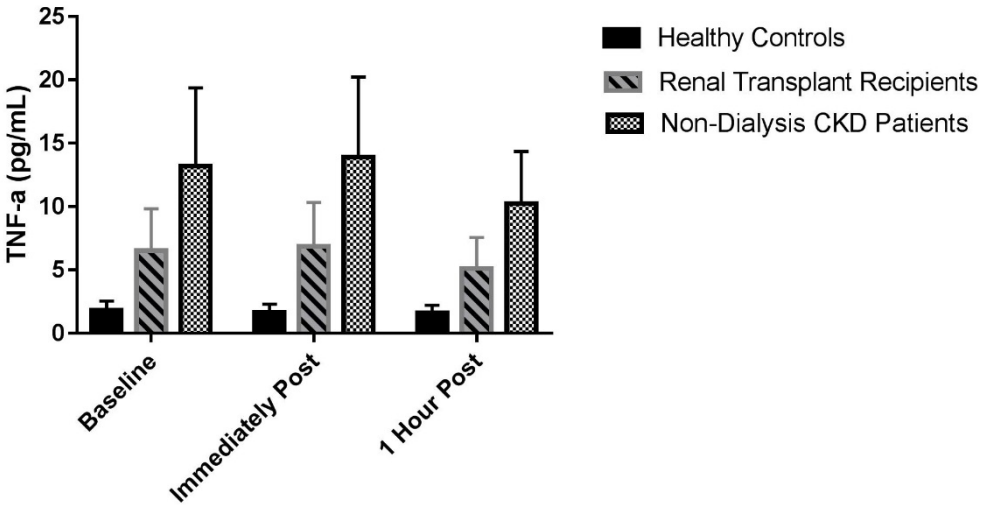

Figure 7.10. Circulation concentrations of TNF-α. Data are presented as 'mean ± SEM'. (HC n = 7, RTR n = 9, CKD n = 9). This data was non-normally distributed and thus was logarithmically transformed prior to analysis.

No significant time*group interaction effect was demonstrated for MIG ($p = .522$, $\eta^2 = .033$). Circulating MCP-1 levels also displayed a significant between-group difference at baseline ($p = .022$), elucidated as a greater concentration in the RTRs than CKD patients ($p = .044$), and a trend towards being greater than in the HC ($p = .052$). MCP-1 also displayed a significant effect of time ($p = .017$, $\eta^2 = .120$) – this effect was characterised by a trend towards reduction from baseline to immediately post-exercise ($p = .052$) and from baseline to 1-hour post-exercise ($p = .053$). No time*group interaction effect was exhibited for MCP-1 ($p = .688$, $\eta^2 = .018$). Finally, circulating IP-10 levels showed no group differences at baseline ($p = .065$), were unaffected by time ($p = .659$, $\eta^2 = .006$) and showed no significant time*group interaction effect ($p = .566$, $\eta^2 = .029$). Post-hoc sample size calculations suggested that a sample size of at least n = 81 would be required to elicit a significant time*group interaction effect in circulating chemokine levels. Circulating MIG and MCP-1 levels in all groups are presented in Figure 7.11.

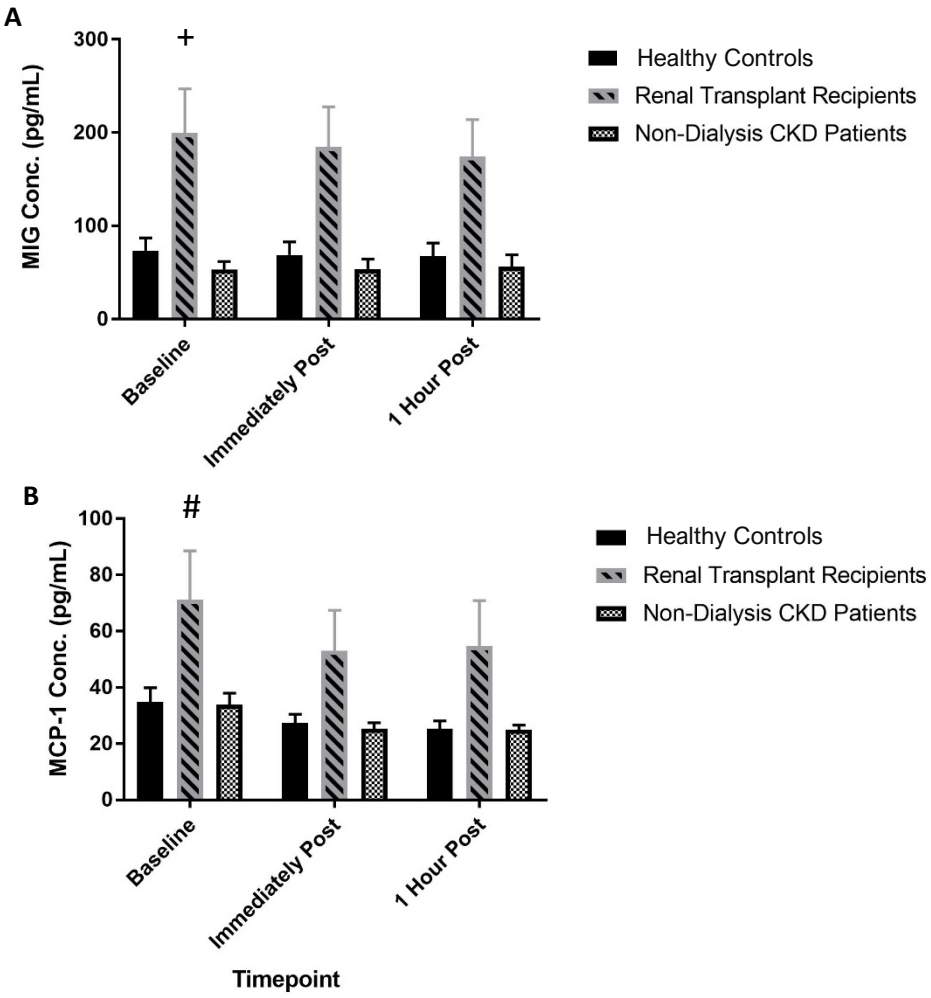

Figure 7.11. Circulating concentrations of the chemokines MIG (A) and MCP-1 (B). Data are presented as 'mean ± SEM'. + = significantly different from the other two populations, # = significantly greater than the CKD population only (only analysed at baseline). (HC = 16, RTR = 15, CKD n = 16). MIG data was non-normally distributed and thus logarithmically transformed prior to analysis, whilst MCP-1 data was normally distributed.

Microparticles

All microparticle data is presented in Table 7.7. A preliminary analysis of baseline CKD patient samples revealed that they did not differ significantly from the baseline RTR samples with regards to total and phenotype specific MP number and MP TF expression. Consequentially, due to the time and financial expenditure of flow cytometric analysis of MPs, full MP analysis was only completed on the HC and RTR populations.

Total MP concentration showed no group differences at baseline ($p = .311$), was unaffected by time and showed no time*group interaction effect ($p = .380$, $\eta^2 = .031$). The number of Annexin-V positive MPs did show a between group difference at baseline ($p = .040$), with the RTRs being greater than the HCs, however was also unaffected by time ($p = .054$, $\eta^2 = .119$) and displayed no significant time*group interaction effect ($p = .257$, $\eta^2 = .053$) .Post-hoc sample size calculations suggested that a sample size of n = 48 would have been required to elicit a significant interaction effect in total MP concentration.

The concentration of platelet-derived MPs and TF^+ platelet-derived MPs exhibited no baseline differences ($p \geq .395$), were unaffected by time ($p \geq .432$, $\eta^2 \leq .025$) and displayed no time*group interaction effect ($p \geq .398$, $\eta^2 \leq .029$). Consequentially, the % of platelet-derived MPs of the total MP pool showed no difference at baseline ($p = .464$), was unaffected by time ($p = .381$, $\eta^2 = .031$) and showed no time*group interaction effect ($p = .375$, $\eta^2 = .032$) (post-hoc sufficient sample size: n = 80). However, the % of platelet-derived MPs that were TF^+, whilst displaying no differences at baseline ($p = .075$) and no time*group interaction effect ($p = .793$, $\eta^2 = .007$), did display a significant effect of time ($p = .010$, $\eta^2 = .229$) – post-hoc analysis revealed that there was a significant decrease from baseline to immediately post-exercise ($p = .001$). These relationships are displayed in Figure 7.12.

	HC (n = 16)			RTR (n = 15)		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**
Total MP conc. ($x10^9$/L)	5.49 ± 10.37	2.50 ± 3.55	1.84 ± 1.40	28.72 ± 76.92	6.26 ± 7.35	5.93 ± 6.50
***AnV$^+$ MP conc.** ($x10^7$/L)	3.51 ± 2.51 #	6.01 ± 2.56	4.67 ± 2.84	7.36 ± 5.69	9.20 ± 6.58	11.12 ± 7.40
***PMP conc.** ($x10^7$/L)	5.10 ± 2.74	3.63 ± 1.58	2.97 ± 1.64	6.18 ± 5.56	2.94 ± 1.70	21.08 ± 73.84
***TF$^+$ PMP conc.** ($x10^7$/L)	2.83 ± 2.19	1.37 ± 0.81	1.31 ± 0.98	3.02 ± 3.73	0.56 ± 0.46	18.68 ± 70.68
% PMP of total MPs	2.26 ± 1.64	2.64 ± 1.43	2.36 ± 2.07	2.12 ± 2.90	1.31 ± 1.55	16.32 ± 56.59
% PMP TF$^+$	51.45 ± 18.24	36.72 ± 12.47 ##	40.67 ± 16.90	37.56 ± 18.91	19.00 ± 7.39 ##	24.88 ± 22.25
***NMP conc.** ($x10^7$/L)	2.08 ± 2.22	1.04 ± 1.21 ##	1.12 ± 1.09 ##	2.17 ± 1.81	1.32 ± 1.27 ##	0.68 ± 1.07 ##
***TF$^+$ NMP conc.** ($x10^7$/L)	0.82 ± 1.11	0.41 ± 0.58	0.32 ± 032 ##	0.49 ± 0.46	0.24 ± 0.28	0.12 ± 0.19 ##
% NMP of total MPs	0.86 ± 1.21	0.82 ± 1.28	0.83 ± 1.18	0.90 ± 1.15	0.49 ± 0.62	0.61 ± 1.40
% NMP TF$^+$	35.59 ± 9.41	33.09 ± 15.76	33.20 ± 20.76 ##	21.68 ± 6.21	18.46 ± 8.94	16.17 ± 6.69 ##
MMP conc. ($x10^7$/L)	2.34 ± 1.43	2.87 ± 2.24	2.15 ± 1.58	2.60 ± 2.09	2.31 ± 1.69	2.19 ± 1.76
TF$^+$ MMP conc. ($x10^7$/L)	0.68 ± 0.49	1.01 ± 0.80	0.72 ± 0.70	0.53 ± 0.51	0.47 ± 0.47	0.32 ± 0.33
% MMP of total MPs	0.97 ± 0.72	2.14 ± 1.82	1.89 ± 1.66 ##	0.69 ± 0.93	0.97 ± 1.29	1.79 ± 2.84 ##
% MMP TF$^+$	28.33 ± 6.67 #	34.74 ± 10.88	32.70 ± 12.98	18.81 ± 8.79	18.57 ± 8.54	14.18 ± 4.92
***EMP conc.** ($x10^7$/L)	0.19 ± 0.38	0.81 ± 0.89	0.53 ± 0.96	3.11 ± 10.09	0.67 ± 0.59	0.98 ± 1.09
***TF$^+$ EMP conc.** ($x10^7$/L)	0.05 ± 0.09	0.02 ± 0.02	0.01 ± 0.02	2.35 ± 0.88	0.09 ± 0.08	0.09 ± 1.10
% EMP of total MPs	0.09 ± 0.17	0.46 ± 0.51	0.46 ± 1.07	1.56 ± 5.48	0.31 ± 0.35	0.52 ± 0.69
% EMP TF$^+$	31.26 ± 11.31	34.49 ± 21.29	20.43 ± 8.25	25.10 ± 30.81	13.18 ± 5.20	9.69 ± 5.54

Table 7.7. All MP phenotype concentration and TF expression data. PMP = platelet-derived MP; NMP = neutrophil-derived MP; MMP = monocyte-derived MP; EMP = endothelial cell-derived MP; AnV$^+$ = Annexin-V-positive MPs. % NMP TF$^+$ = % of total NMP pool that positively express TF. * indicates that data was non-normally distributed and thus was logarithmically transformed prior to analysis. # = significant difference between groups. ## = significantly different from baseline (main effect of pooled data). Data are presented as 'mean ± SD' (HC n = 16, RTR n = 15).

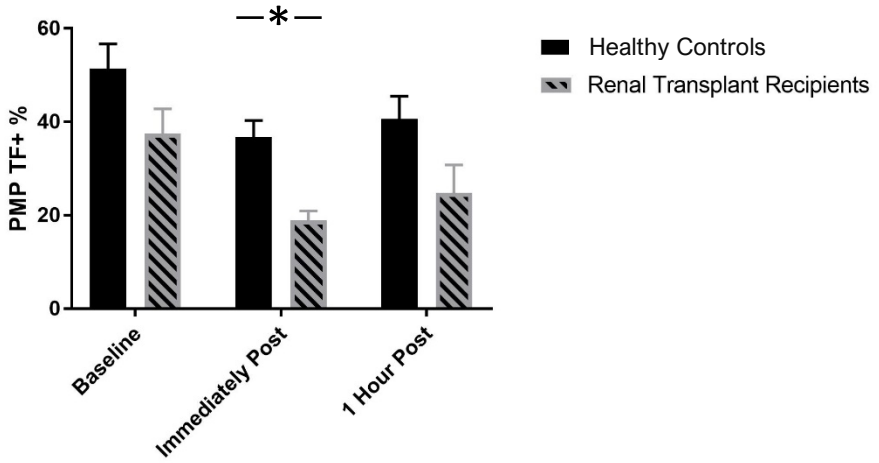

Figure 7.12. The percentage of platelet-derived microparticles (PMPs) that positively express tissue factor (TF). Data are presented as 'mean ± SEM'. * = significantly different from baseline. (HC n = 16, RTR n = 15). This data was normally distributed.

The concentration of neutrophil-derived MPs displayed no between-group difference at baseline ($p = .909$), however was significantly affected by time ($p = .004$, $\eta^2 = .222$) – post-hoc analysis revealed that this was due to a significant reduction in neutrophil-derived MP concentration from baseline to immediately post-exercise ($p = .047$) and from baseline to 1-hour post-exercise ($p = .013$). However, there was no time*group interaction effect ($p = .518$, $\eta^2 = .024$). Consequentially, the concentration of TF^+ neutrophil-derived MPs also displayed no group difference at baseline ($p = .304$), however was also significantly affected by time ($p = .012$, $\eta^2 = .186$), characterised by a reduction from baseline to 1-hour post-exercise only ($p = .024$). As with neutrophil-derived MP concentration, there was also no time*group interaction effect ($p = .757$, $\eta^2 = .008$). The neutrophil-derived MP concentrations and TF expressions are presented in Figure 7.13. Contrasting with this, the % of neutrophil-derived MPs of the total MP pool and the % of neutrophil-derived MPs that were TF^+ showed no between-group differences at baseline ($p \geq .063$), no main effect of time ($p \geq .670$, $\eta^2 \leq .044$) and no time*group interaction effect ($p \geq .692$, $\eta^2 \leq .071$) (post-hoc sufficient sample size: n = 36).

The concentration of circulating monocyte-derived MPs showed no group differences at baseline ($p = .720$), was unaffected by time ($p = .563$, $\eta^2 = .023$) and displayed no time*group interaction effect ($p = .582$, $\eta^2 = .021$). The concentration of TF^+ monocyte-derived MPs also showed no baseline difference (p

= .443), was unaffected by time (p = .144, η^2 = .075) and displayed no significant time*group interaction effect (p = .216, η^2 = .059). Whilst the % of monocyte-derived MPs showed no between-group difference at baseline (p = .394), there was a significant affected of time (p = .033, η^2 = .137), with a significant increase from baseline to 1-hour post-exercise (p = .045). However, there was time*group interaction effect (p = .303, η^2 = .046). Conversely, the % of monocyte-derived MPs did display a between-group difference at baseline (p = .006) (HCs elevated relative to RTRs), though there was no effect of time (p = .418, η^2 = .049) or significant time* group interaction effect (p = .126, η^2 = .129).

Lastly, the endothelial cell-derived MP concentration, TF^+ endothelial cell-derived MP concentration, % of MPs that were endothelial cell-derived MPs and the % of endothelial cell-derived MPs that were TF^+ showed no group differences at baseline (p ≥ .287), remained unchanged by time (p ≥ .200, η^2 ≤ . 124) and showed no significant time*group interaction effects (p ≥ .229, η^2 ≤ .059) (post-hoc sample size analysis suggested that n = 44 would be required to detect a significant interaction effect).

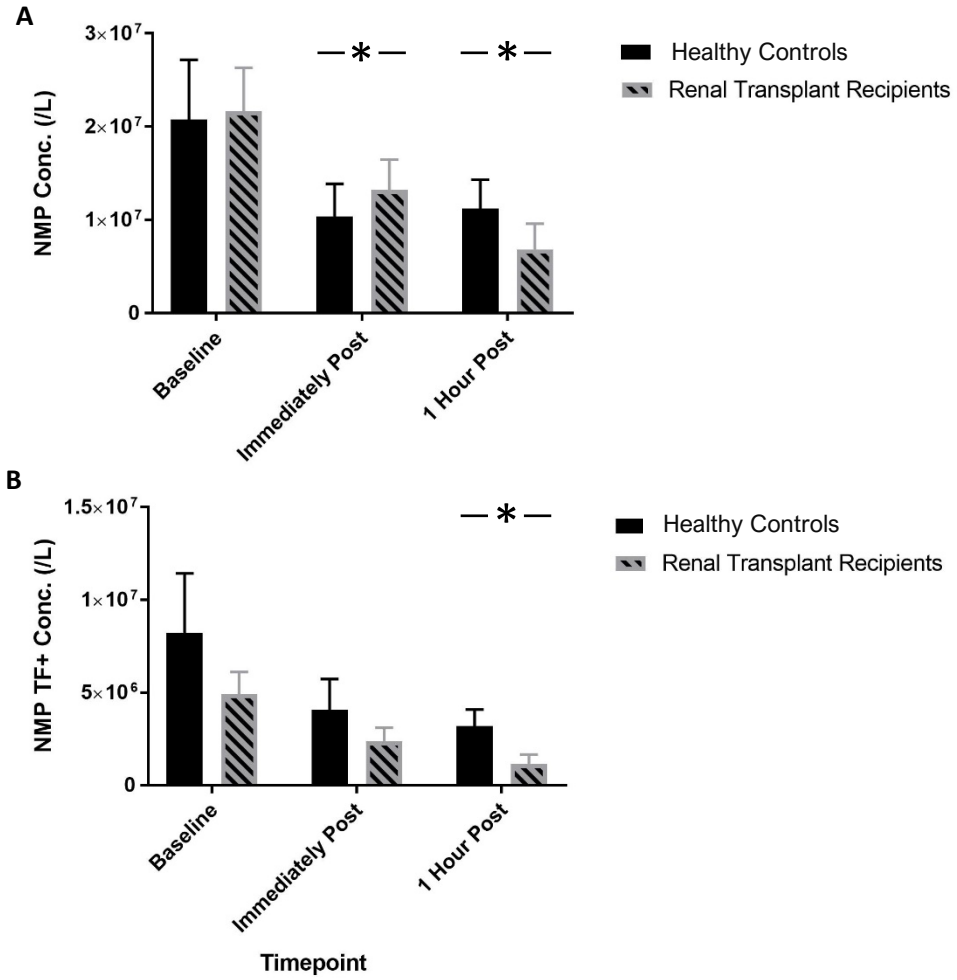

Figure 7.13. Circulating concentration of neutrophil-derived microparticles (NMPs) (A) and tissue factor-positive (TF+) neutrophil-derived MPs (B). Data are presented as 'mean ± SEM'. * = significantly different from baseline. (HC n = 16, RTR n = 15). This data was non-normally distributed and thus subject to logarithmic transformation prior to analysis.

7.5 Discussion

The aim of this study was to investigate the impact of a single 20-minute bout of moderate intensity walking exercise on immune cell phenotypes and circulating cytokines, chemokines and MPs in healthy control participants, RTRs and non-dialysis dependent CKD patients. Whilst the exercise bout decreased the circulating percentage of the intermediate and non-classical monocyte subsets and increased the classical subset, there were no other effects of exercise on immune cell subsets, though several measures of immune cell subset composition differed between groups at baseline. Circulating pro- and anti-inflammatory cytokines were largely unaffected by the exercise, however showed trends towards significant differences between groups, whilst circulating chemokine levels were also unresponsive to the exercise bout but did exhibit differences between groups. Lastly, whilst the absolute number of most cell-derived MPs remained unchanged, there were significant differences seen in response to time as an effect of exercise in the characteristics of several MP phenotypes. These relationships are discussed in detail below.

Demographics and clinical information

The three populations were well matched when considering all demographic (i.e. age, gender, ethnicity) and anthropometric/body composition (i.e. BMI, body fat %, skeletal muscle mass). This is perhaps surprising, as CKD populations, particular those who are also taking immunosuppressive medication, have been shown to have worsened body composition profiles when compared to the general population, characterised by a reduction in skeletal muscle mass and an increase in body fat mass (Mafra, Guebre-Egziabher, and Fouque 2008). In the case of renal transplantation, this is largely caused by both the removal of the strict dietary requirements integral to dialysis treatment (Uysal et al. 2015) and the catabolic effect of traditional immunosuppressive treatment regimens (Ekstrand et al. 1996). However, there were no significant differences between the populations in this study.

Unsurprisingly, hypertension was much more prevalent in the renal disease populations. Hypertension can be both a primary cause of, and secondary to, CKD (either essential hypertension causes CKD by damaging the small blood vessels within the nephron, or the reduced filtering capacity of the diseased

kidneys results in fluid overload and hence secondary hypertension). As such, hypertension is highly prevalent in CKD populations (MacNeill et al. 2018). However, it is perhaps surprising that there were no other significant differences observed with regards to comorbidity, particularly in the case of diabetes which is highly prevalent in CKD populations (MacNeill et al. 2018) and can display a similar cause-and-consequence relationship to hypertension.

The clinical laboratory results clearly demonstrate that the transplant and CKD populations used in this study truly represent a renal disease population when compared to the HC group, characterised by a significant reduction in eGFR, and significantly elevated circulating urea and creatinine measures. Similarly, the significantly lower platelet count in the renal transplant group is as expected as several immunosuppressive drugs (e.g. sirolimus, cyclosporine, prednisolone) have been shown to reduce platelet count in a dose-response manner (Murgia, Jordan, and Kahan 1996). Inhibition of platelet proliferation during haematopoiesis has been proposed as the mechanism behind this relationship. However, the significantly increased platelet count in the CKD population is unexpected – whilst measures of platelet activation (e.g. GPIIb/IIIa expression) have been shown to be elevated in CKD populations, absolute platelet counts tend to be similar to the general population (Gremmel et al. 2013).

Cardiovascular function and physical activity levels

The lack of differences seen in cardiovascular function measures (cardiac output, stroke volume, total peripheral resistance) is unusual given the general systematic pathophysiological effects of CKD on the cardiovascular system (Schiffrin, Lipman, and Mann 2007). However, the prevalence of cardiovascular co-morbidities that are typically common in CKD populations (e.g. ischaemic heart disease) was relatively low in the renal transplant (6.7%) and CKD patients (12.5%). As such, the patient populations may have not yet developed any cardiac impairment significant enough to be measurable using a NICOM system. Similarly, the lack of differences seen in weekly physical activity levels is interesting as CKD populations typically display significantly reduced levels of habitual physical activity when

compared to the general population (Beddhu et al. 2009). However, this may simply be the product of recruitment bias; patients who are typically more habitually active may be more likely to participate in research involving exercise and physical activity than their more sedentary counterparts. Additionally, this was a self-reported measure which can therefore be subject to various biases such as social desirability and general misreporting.

Physical performance

The HC population achieved a significantly greater distance in the ISWT than the renal patient populations. This was an expected finding – CKD patient populations display significantly reduced functional capacity when compared to the general population and hence typically display lower scores on physical function or VO_2 peak tests (Johansen 2005). It is therefore also unsurprising that the HC participants reported a lower post-ESWT RPE, despite post-ESWT heart rates being similar among the groups.

Immune cell phenotypes

The increase in circulating neutrophil concentration throughout the trial is unsurprising – previous research has shown significant neutrophilia following acute aerobic exercise of various intensities (Quindry et al. 2003; Robson et al. 1999). This is most likely due to increased neutrophil demargination caused by both increased haemodynamic stress and increased circulating catecholamine concentration. The increased neutrophil concentration in the CKD population (trend towards increase in RTRs) at baseline is relatively harder to explain as there is limited previous research investigating this relationship. However, a possible explanation is an alteration is the way that neutrophils are cleared from the circulation. Uraemic toxins and retention solutes may reduce the bactericidal capacity of neutrophils (Kato et al. 2008). In response to this, in an attempt to regain some of the lost host defence capabilities, neutrophil apoptosis is impaired in order to increase the available neutrophil pool, thus elevating the circulating neutrophil concentration. However, this may not be a desirable effect, as the longer a neutrophil remains in the circulation the more likely it is to undergo un-programmed necrosis (rather than programmed and controlled apoptosis) and initiate a pro-inflammatory cascade that

contributes to chronic systemic low-grade inflammation (Kato et al. 2008). Therefore, it seems there is a balance to be struck, i.e. maintaining the available neutrophil pool to bolster innate immune defences whilst not significantly increasing the rates of neutrophil necrosis.

Whilst no changes were seen in the absolute number of total or subset-specific monocytes, significant variations were observed in the percentage of these subsets within the total cell population. The percentage of classical ($CD14^{++}CD16^{-}$) monocytes was increased throughout the trial in all three groups, seemingly at the expense of the intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) subsets which both displayed an overall decrease from pre to post-exercise. This subset shift should represent an anti-inflammatory effect – classical monocytes are largely considered to be anti-inflammatory as they produce the largest amount of IL-10 and the least amount of IL-6, whilst $CD16^{+}$ monocytes (i.e. intermediate and non-classical) are deemed to be primarily pro-inflammatory as they can produce large amounts of TNF-α and IL-1β (Wong et al. 2012). Additionally, $CD14^{++}CD16^{+}$ intermediate monocytes have been independently associated with cardiovascular events in CKD patients (Rogacev et al. 2011). As such, a shift towards the classical subset and away from the $CD16^{+}$ subsets should be considered a favourable subset shift. Whilst no changes were observed in the absolute numbers of each subset, it is more likely that the relative composition and thus balance of pro- vs anti-inflammatory influence is more clinically significant than the absolute numbers of each subset, however further research is needed to confirm this. These results are in contrast to those seen by Van Craenenbroeck et al, who found a relative increase in intermediate and non-classical subsets and a relative decrease in the classical subset following acute aerobic exercise in non-dialysis CKD patients (Van Craenenbroeck et al. 2014). However, the exercise intensity may be the cause for this disparity – Van Craenenbroeck et al employed a maximal cardiopulmonary exercise test, whilst this study used only moderate intensity walking exercise and varying aerobic exercise intensities can induce significantly different leukocyte responses (Neves et al. 2015).

RTRs have been found to display expansion of the $CD16^{+}$ subsets when compared to the general population (Vereyken et al. 2013). As such, the between-group differences in the classical and

intermediate subset percentages found in this study are interesting. The increased percentage of intermediate monocytes in the CKD patients only may reflect innate immune activation by unfiltered uraemic toxins due to reduced renal function, and the similar levels seen in the RTRs compared with the HCs suggests that the immunosuppressive medication is hindering this activation and may represent a protective effect. However, immunosuppressive medications taken by transplant recipients typically target lymphocyte activation and proliferation rather than monocytes directly, and as such this effect requires further investigation. Similarly, the increased percentage of classical monocytes seen only in the RTRs may also suggest that the immunosuppressive medication is hindering $CD16^{+}$ monocyte development, however there is no currently proposed mechanism for this.

Monocyte ACE (angiotensin-converting enzyme – elicits conversion of ACE I to ACE II) expression has been associated with cardiovascular risk via ACE II-induced activation of the renin-angiotensin-aldosterone system (RAAS), leading to vasoconstriction, oxidative stress and inflammation (Ferrario and Strawn 2006), ultimately leading to arterial intima-media thickening and atherosclerosis. CKD populations have been found to display elevated levels of monocyte ACE expression (Trojanowicz et al. 2016), which has been linked with increased rates of atherosclerosis (Trojanowicz et al. 2016) and ultimately increased cardiovascular disease prevalence (Ulrich et al. 2006) and cardiovascular mortality (Ulrich et al. 2010). To the author's knowledge, this is the first study to investigate the impact of any kind of exercise on monocyte ACE expression. Whilst the RTRs displayed an increased number of circulating ACE^{+} monocytes and reduced mean monocyte ACE GMFI, there was no significant impact of exercise on monocyte ACE expression. As has been shown in this study, and in previous research (Van Craenenbroeck et al. 2014), acute aerobic exercise can impact the composition of monocyte subsets, however acute aerobic exercise does not seem to impact monocyte ACE expression. Whilst previous murine research has suggested that regular aerobic exercise training can reduce ACE activity (Pereira et al. 2009), it seems that this effect is not mediated by ACE that is expressed by monocytes, at least not at the intensity studied here. Whilst this suggests that monocyte ACE expression is not an avenue by which aerobic exercise can reduce cardiovascular risk, it is encouraging that there was no exercise-induced aberrant activation that may exert detrimental effects.

Typically, as a result of the increased blood flow and catecholamine release in response to aerobic exercise, demargination of lymphocytes occurs which significantly increases the number of lymphocytes present in the circulation. As such, the lack of changes in total lymphocyte number observed in this study is unusual, especially given that this relationship was observed in the neutrophils assessed in this study. However, in previous research neutrophils have proven much more responsive to acute aerobic exercise than lymphocytes, with neutrophilia being responsible for the large majority of exercise-induced leukocytosis and lymphocytosis contributing relatively less (Sand et al. 2013). Additionally, as the exercise completed here was of only moderate intensity and short duration, it may simply be that the total volume of exercise was not sufficient to induce significant lymphocyte demargination and thus lymphocytosis was not observed.

However, significant differences and changes were observed in the relative lymphocyte subset composition. The greater proportion of T-regs in the total lymphocyte pool in the HCs is in line with previous research which has found reduced numbers of circulating T-regs in CKD populations (Hendrikx et al. 2009). The increase in proportion of circulating T-regs seen in all populations tested here in response to exercise is encouraging as it suggests an anti-inflammatory effect of exercise, as has been observed elsewhere (Gleeson et al. 2011), and would be expected to result in increased rates of secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. This increase is also of particular importance to the RTRs, as $CD4^+CD25^+$ T-regs (as they have been assessed in this study) are integral to the active maintenance of immunological self and non-self-tolerance, and are thus vital for graft tolerance and the long-term health of the transplanted kidney (Sakaguchi 2005). However, as with the monocytes, there was no change in the absolute number of circulating T-regs and this may be more clinically important.

The exercise-induced decrease in circulating cytotoxic ($CD3^+CD8^+$) T cells during the recovery period is unusual – typically as these more terminally differentiated cells express a higher density of β2-adrenergic receptors, they are more responsive to exercise-induced catecholamine increases than helper ($CD3^+CD4^+$) T cells and thus would be expected to display an exercise-induced increase. However,

as before, this may be a product of insufficient exercise intensity and thus the decrease may be due to other factors, for instance diurnal variation which can potently impact leukocyte subset distribution (Born et al. 1997; Suzuki et al. 1997). Given the lack of changes in total lymphocyte number, it is unsurprising that no other changes were observed in the absolute circulating number of any other the other lymphocyte subsets assessed here.

Lastly, there was an overall increase in circulating B cell % throughout the trial (despite there being a reduction from baseline to immediately post-exercise). Aside from the effects on demargination as mentioned earlier, a possible explanation for this may be the composition of the helper T cells. Type 2 helper T cells produce mainly IL-4, IL-5, IL-10 and IL-13 and induce a shift towards humoral (i.e. B cell) immunity and away from cell-mediated (i.e. T cell) immunity. Whilst helper T cell phenotypes were not assessed in this study, previous research has revealed a relative reduction in Type 1 helper T cell % and no change in Type 2 % (Gleeson 2007), which suggests a relative shift away from cell-mediated immunity and towards humoral immunity. This would therefore explain the relative increase in B cell %, however this relationship requires further investigation.

Circulating cytokines

The HC population displayed a trend towards a reduced resting concentration of circulating IL-6. The relatively elevated circulating IL-6 concentration in the RTR and CKD populations is unsurprising – the elevated levels of uraemic toxins (as demonstrated in Table 7.1) would act as ligands for leukocytes and thus cause immune cell activation and subsequent proliferation and pro-inflammatory cytokine production. Additionally, when considering Figure 7.3, the HC population had the lowest circulating percentage of intermediate monocytes, which have been suggested to be a pro-inflammatory subset and to secrete the greatest amount of IL-6 relative to the other two subsets (Rossol et al. 2012). Elevated circulating levels of IL-6 in renal patient populations have been displayed in previous research (Oberg et al. 2004). Whilst IL-6 can also be released from the working muscle (then deemed a 'myokine') (Pedersen and Febbraio 2008), skeletal muscle mass did not differ between the three populations. As such, as the skeletal muscle mass did not differ between the groups, the amount of IL-6

released from this skeletal muscle likely did not differ between the groups, and as such IL-6 released from skeletal muscle mass is unlikely to have contributed to this effect. Similarly, this mechanism is also governed by the skeletal muscle contraction force (mediated by alterations in calcium release) – similar relative exercise intensities were achieved in this study so this is also unlikely to be a factor. Circulating IL-10 concentrations at rest displayed no differences between groups. IL-10 has been shown to be highly responsive to IL-6 levels – any transient increase in circulating IL-6 results in a delayed increase in circulating IL-10 (Steensberg et al. 2003). As such, it is surprising that IL-10 levels were not elevated in response to the IL-6 levels in the patient groups. However, as there was only a trend towards an increase in IL-6 in these groups (that did not reach statistical significance), it is possible that the IL-6 stimulus was not sufficient to induce a detectable response in resting circulating IL-10 concentrations.

The general lack of effect of exercise on circulating IL-6 and IL-10 levels is perhaps more surprising (despite there being an interaction effect observed in IL-10, meaning the RTRs displayed significantly elevated circulating values at 1-hour post-exercise than the HCs). In a similar study, Viana et al found that 30 minutes of moderate intensity (RPE 12-14) walking exercise in non-dialysis dependent CKD patients induced a significant increase in both IL-6 and IL-10 circulating levels 1-hour post-exercise (Viana et al. 2014). As explained above, this was most likely caused by IL-6 release from the working muscle causing a subsequent rise in IL-10 secretion. It is unclear why the same effect was not shown in this study - the exercise was of the same modality and of similar intensity (12-15 depending on the population). However, the duration implemented here (20 mins) was shorter than that employed by Viana et al (30 mins) and thus it may simply be that the volume of exercise was not sufficient to stimulate significant cytokine secretion.

Circulating TNF-α and IL-17a levels were also unaffected by exercise (though the reduced sample sizes assessed for these cytokines should be noticed due to the low number of samples displaying values above the minimum detectable threshold of the technique – IL-17a n = 13/47, TNF-α n = 25/47). Whilst TNF-α may be responsive to long term exercise training, potentially via alterations in adipocyte or macrophage phenotypes, following acute exercise it seems largely to be dependent on IL-6 levels in a

similar manner to IL-10 (Gleeson et al. 2011; Pedersen and Febbraio 2008). As no exercise-dependent effects were seen in IL-6, it is therefore unsurprising that TNF-α also remained unchanged. Lastly, there is very little research regarding IL-17a and its relationship with exercise. IL-17a is a pro-inflammatory cytokine that is secreted by Th17 lymphocytes – these cells act in an opposing manner to T-regs in that they promote immune activation and have been implicated in autoimmune conditions, whilst also providing host defence against infectious diseases (Cortvrindt et al. 2017). Whilst strenuous endurance exercise protocols (e.g. marathon or triathlon events) in trained athletes have been shown to elicit increases in circulating Th17 cells and IL-17a concentration (Perry et al. 2013; Sugama et al. 2012), less is known about the effects of lower intensity exercise in untrained or clinical populations. It may be that exercise of a sufficient volume or intensity, or exercise which induces significant muscle damage is required to increase the circulating concentration of this cytokine, as has been previously suggested (Brunelli and Rovere-Querini 2008) and that the level of exercise intensity that an untrained, chronic disease patient is able to achieve is far lower than that required to induce a measurable increase in circulating IL-17a concentration. However, as has been mentioned in previous studies, a more targeted approach to measuring circulating IL-17a concentrations may be necessary to elucidate its possible clinical significance, particularly as elevated resting circulating IL-17a levels have been associated with hypertension and atherosclerosis in CKD populations (Cortvrindt et al. 2017), therefore warranting further investigation

Circulating chemokines

Chemokines are a subset of cytokines that elicit chemotaxis in nearby cells that express the appropriate chemokine receptor. Under normal physiological conditions, chemokines help to combat infections or initiate would healing by attracting leukocytes to the site of injury. However, chemokine secretion can also be induced by pro-inflammatory cytokines (e.g. IL-1, TNF-α) and as such can be persistently elevated during chronic systemic inflammation (Graves and Jiang 1995). Whilst there has been little research investigating the impact of aerobic exercise on chemokine kinetics, the small body of literature suggests that strenuous acute exercise (a marathon) increases the circulating concentrations of various

chemokines (IL-8, MIP (macrophage inhibitory protein)) in healthy individuals (Ostrowski et al. 2001), whilst 12-weeks of regular, moderate intensity combined endurance and resistance training in individuals with metabolic syndrome can reduce the resting circulating concentrations of MCP-1 and IL-8 (Trøseid et al. 2004).

In this study, the measured chemokines were largely unresponsive to the exercise trial. This is unsurprising, given that the intervention assessed by Ostrowski et al was of significantly greater duration and intensity (i.e. a marathon) than the intervention employed here. As increases in IL-6 have been implicated as a stimulator for chemokine secretion and there was not a significant increase in IL-6 concentration in this study, it stands to reason that there was no subsequent chemokine response.

However, both MIG (monokine induced by gamma interferon – CXCL9, a T cell attractant) and MCP-1 displayed significant differences between groups in this study. Surprisingly, despite the non-dialysis CKD patients tending to exhibit the greatest circulating IL-6 levels, the RTRs displayed significantly greater circulating concentrations of MIG and MCP-1 than the other two populations. MIG is primarily induced by interferon gamma (IFN-γ) which was not assessed in this study, which makes explaining this effect difficult. However, MIG is a chemokine that specifically targets T lymphocytes. As the immunosuppressive regime required by renal transplantation is specifically designed to deplete primarily cytotoxic T lymphocytes (by inhibiting both cellular activation and proliferation and intracellular energy metabolism), it is logical to suggest that MIG secretion would be elevated as a compensatory response to the lack of circulating T cells. Whilst there is limited research regarding MIG in ESRD, elevated urinary MIG has been associated with acute rejection episodes in RTRs (Hauser et al. 2005) and as such it represents an interesting target for future research in this population. However, the MIG values seen in the RTRs in this study, whilst greater than those of the HCs and CKD patients (175-200 pg/mL vs 50-75 pg/mL), are significantly lower than those seen in previous research investigating acute rejection, which recorded values over 1,000 pg/mL and sometimes as high as over 10,000 pg/mL in patients who subsequently experienced rejection episodes (Hauser et al. 2005). As such, there is little cause for concern here. Albuminuria has been positively associated with MCP-1-induced renal injury,

with greater degrees of albuminuria being associated with greater concentrations of urinary MCP-1 (Eardley et al. 2006). As such, reduced renal function and the consequential albuminuria would be expected to coincide with elevated circulating MCP-1 concentrations. However, in this study, the HCs (mean eGFR of 86 mL/min/1.73m^2) and the non-dialysis CKD patients (mean eGFR of 62 mL/min/1.73m^2) displayed similar circulating MCP-1 concentrations. Similar to MIG, elevated MCP-1 concentrations have been associated with acute rejection episodes in RTRs. Krüger et al demonstrated that circulating MCP-1 levels may be elevated in certain RTRs due to a specific gene polymorphism, and that peripheral blood mononuclear cells isolated from patients with this gene polymorphism produced a 2.5 fold greater IL-1β-stimulated MCP-1 secretion rate than those without it (Kruger et al. 2002). The presence of this polymorphism was also significantly associated with reduced allograft survival. However, as with MIG, circulating MCP-1 concentration was only marginally elevated in the RTRs when compared to the HC and CKD populations (50-70 pg/mL vs 25-35 pg/mL) and previous research concerning rejection episodes has reported MCP-1 values of 200-300 pg/mL and suggested that MCP-1 values may not actually rise significantly when compared to other chemokines (e.g. RANTES) (Corsi et al. 1999), again meaning there is little cause for alarm here. Finally, ACE-inhibitor treatment has been shown to attenuate renal expression of MCP-1 (Hilgers et al. 2000). As the number of all monocyte subsets expressing ACE was significantly elevated in the RTRs only, this may provide a possible mechanism for the elevated MCP-1 circulating concentrations seen in this study. As elevated MCP-1 concentration has been implicated in the initiation and progression of renal injury and disease (e.g. via promotion of tubulointerstitial damage) (Viedt and Orth 2002), this relationship represents a meaningful target for future research.

Circulating microparticles

As explained in Chapter 2, the increased blood flow induced by aerobic exercise increases vascular shear stress and thus results in increased MP shedding. However, there was no effect of exercise on total MP number in this study. The moderate exercise intensity, combined with the large observed degree of inter-patient variation in MP numbers, may explain why no changes were seen here. Similarly, the so-

called ‘pan-cell’ marker for MPs, Annexin-V (as a marker of phosphatidylserine (PS) expression), was unchanged by exercise. However, the RTRs displayed a significantly greater circulating concentration of Annexin-V^+ MPs than the HCs. Previous research has suggested that the initiating mechanism behind MP formation may influence the subsequent degree of PS externalisation. Specifically, it has been suggested that when MPs are formed during cellular apoptosis (as opposed to merely cellular activation or stress) they externalise a greater amount of PS in their phospholipid membrane (Jimenez et al. 2003) and thus bind with Annexin-V to a greater degree. This hints towards a greater degree of cellular apoptosis in the RTRs, however this is only speculative. Future research should include measures of apoptotic bodies as well as MPs to attempt to explain this relationship.

As there was no significant change in total MP number, it is unsurprising that there were no significant changes in the majority of the measured MP phenotype numbers (platelet-derived MPs, monocyte-derived MPs, and endothelial cell-derived MPs). However, it is unclear why only neutrophil-derived MPs were significantly reduced following exercise. This suggests a specific exercise-induced mechanism that would only influence neutrophils and leave platelets, monocytes and endothelial cells unaffected. This likely rules out any haemodynamic influence and suggests a more cell-targeted effect. Another possible explanation is an alteration in the number of circulating neutrophils. However, circulating neutrophil concentration was increased in all groups throughout the trial. A possible explanation is a shift in neutrophil phenotype – as only one marker (CD66b) was used to identify neutrophil-derived MPs, a phenotype shift may have occurred which resulted in reduced CD66b expression (Galli, Borregaard, and Wynn 2011) rather than reduced neutrophil-derived MP release, artificially lowering the concentration when using the protocol implemented in this study. However, as neutrophils were not included in the leukocyte flow cytometry panel and the haematology analyser used cannot distinguish neutrophil phenotypes, it is difficult to explain this relationship. The decrease in the number of TF^+ neutrophil-derived MPs is most likely simply as a result of the decline in total neutrophil-derived MP number, particularly as the neutrophil-derived MP TF^+ % remained unchanged.

However, exercise did induce a decrease in the circulating % of TF^+ platelet-derived MPs. Platelets are integral in thrombosis, and ESRD patients have displayed a greater tendency towards thrombotic events due to increased platelet aggregation and pro-coagulant activity (Kaw and Malhotra 2006). Platelet-derived MPs are now thought to be a secondary mediator of this effect – *in vitro* studies have positively correlated platelet-derived MP concentration with endogenous thrombin potential (Macey, Enniks, and Bevan 2011). Similarly, MP TF expression has been highly implicated in thrombus formation (Polgar et al. 2005). As such circulating platelet-derived MPs with pro-thrombotic potential have been suggested to trigger thrombosis in ESRD patients (Ando et al. 2002). Therefore, the reduction in TF^+ % represents a positive effect. However, as the absolute number of TF^+ platelet-derived MPs was unchanged by exercise, it remains to be seen if this represents a clinically significant effect. As with the immune cell data, it is not yet clear if relative or absolute concentrations will determine the pathophysiological influence. Additionally, the platelet-derived MP TF^+ % in the RTRs is slightly unexpected, as ESRD patients typically display an increased number of pro-coagulant MPs when compared to the general population (Burton et al. 2013). However, the majority of this research has occurred in dialysis patients (Trappenburg et al. 2012). Renal transplantation reduces the number and activity of pro-coagulant platelet-derived MPs within the first year post-transplantation, however not to the level seen in the general population (Al-massarani et al. 2009). As the current transplant duration of the RTRs in this study was largely greater than one year (6.25 years), this may explain the further reduced platelet-derived MP TF^+ %. However, as this only reached significance immediately post-exercise, it is unclear if this represents an actual chronic change or simply a transient effect of exercise. As a similar relationship was observed in the monocyte-derived MP TF^+ % (i.e. higher in the HCs), this warrants further investigation. Whilst only speculative at this point, it may be that the immunosuppressive medication is impacting MP protein expression.

Limitations

Firstly, whilst immune cell subsets were assessed in this study, no direct measures of immune function were included. As such, any variations seen in leukocyte number or distribution can only be used to

describe the immune environment rather than directly comment on immune function. Secondly, there was no non-exercise visit included in this study design. Whilst the study design included a healthy control condition (and CKD patients as a uraemic control), the effects of exercise vs rest cannot be investigated. Lastly, the laboratory analysis techniques employed displayed limitations, such as the inability of the cytometric bead array technique to detect IL-2 and to a large extent IL-17a and TNF-α, and the large coefficient of variations presented for this technique and the MP analysis technique (10.08% and 13.88%, respectively), which likely increased the chance of a type 2 error.

Conclusions and future research

In summary, an acute bout of moderate intensity walking exercise elicited compositional changes in immune cell phenotypes, little change in circulating cytokines and chemokines and a reduction in the circulating % of TF^+ MPs. Whilst between-group differences were seen in these measures, the most important finding is that the exercise did not induce drastic changes in immune cell phenotypes or in inflammatory markers. As such, this sort of exercise should be considered safe for this patient population from an immunological perspective, and they should be encouraged to participate in regular moderate intensity exercise just as the general population is. Whilst the 'correct amount' of immune function for RTRs is unclear (i.e. over-suppression that leads to increased infection rates vs under-suppression that risks graft loss), this exercise modality and intensity seems to not impact this relationship. Future research should focus on varying exercise intensities and modalities in order to investigate their efficacy and 'immunological safety' in order to create and inform future exercise guidelines for RTRs.

Chapter 8

General Discussion

8.1 Results summary

The primary aim of this thesis was to investigate the effects of both acute and long-term aerobic exercise on markers of systemic inflammation, immune function and MP phenotypes in end stage renal disease populations. This was accomplished by: characterising the MP phenotype response to aerobic exercise in healthy individuals in Study 1; investigating the impact of 6-months of intradialytic exercise on markers of systemic inflammation and MP phenotypes in Study 2; comparing the effects of 12 weeks of intradialytic cycling training and low-frequency electrical muscle stimulation on markers of systemic inflammation in haemodialysis patients in Study 3; and investigating the effects of an acute bout of aerobic exercise on markers of systemic inflammation, immune function and MP phenotypes in RTRs in Study 4.

In Study 1 (Chapter 4), a single 1-hour bout of moderate intensity aerobic exercise did not significantly alter total or phenotype-specific MP concentrations, nor did it affect the absolute number of MPs positively expressing TF. However, the proportion of platelet and neutrophil MPs that positively expressed TF was significantly reduced in the exercise trial only. This suggests that aerobic exercise may reduce the ability of MPs to induce thrombosis and thus represents a therapeutic effect. Whilst MPs assessed by nanoparticle tracking did not respond to time or trial, the mean diameter results suggest that adding NTA to flow cytometry in MP analysis protocols allows the assessment of the entire size range of MPs, and provides the greatest amount of total information.

In Study 2 (Chapter 5) the 6-month IDC intervention did not alter clinical characteristics and failed to induce a significant increase in measures of physical performance. However, the exercise group did display a reduction in the circulating concentration of the pro-inflammatory cytokine TNF-α, suggesting the exercise had an anti-inflammatory effect. All measured circulating chemokines were unaffected, suggesting that the ability to induce chemotaxis in leukocytes was unaltered. Lastly, the IDC intervention did not impact circulating MP phenotype concentrations or TF expression.

In Study 3 (Chapter 6), both IDC and intradialytic LF-EMS elicited a significant increase in VO_2 peak and max load achieved during a progressive exercise test. This is encouraging as it suggests a possible

alternative exercise modality for patients who are unable to complete dynamic exercise. However, no changes were seen in any measured inflammatory markers. This is unusual given the positive effects observed in Study 2, especially when considering the mean session intensity was greater in Study 3 than in Study 2. Therefore, this was most likely due to the large degree of inter-patient variation observed in this study.

In Study 4 (Chapter 7), an acute bout of moderate intensity walking exercise elicited a shift away from the potentially pro-inflammatory intermediate monocyte subset to the same degree in RTRs, non-dialysis dependent CKD patients and healthy controls. The same relationship was revealed for the expression of TF on platelet-derived MPs, suggesting that the exercise elicited a reduction in the pro-thrombotic potential of these particles. Whilst exercise did not affect any other measured parameters, significant differences between groups were observed. The patient populations displayed a trend towards a circulating concentration of IL-6 and a reduction in T-reg number, suggesting a shift towards a more pro-inflammatory environment than the healthy controls. Additionally, the RTRs displayed an increased number of monocytes that positively expressed ACE but a reduced mean ACE-expression intensity. Lastly, the RTRs displayed elevated circulating levels of several chemokines (MIG and MCP-1), suggesting increased ability to induce leukocyte chemotaxis and tissue infiltration and thus promote endothelial dysfunction and atherosclerosis.

These studies add to the current body of literature concerning systemic inflammation in end stage renal disease patients, and provide novelty by combining analysis of circulating cytokines, chemokines, immune cell phenotypes and microparticles and investigating the effects of novel, non-traditional exercise modalities.

8.2 Inflammation

The various exercise modalities investigated in this thesis largely had little impact on circulating markers of systemic inflammation (i.e. pro- and anti-inflammatory cytokines). Aside from Study 2 (Chapter 5), in which the exercise group displayed a reduction in TNF-α throughout the intervention, no other effects of exercise were exhibited. Whilst at first this may seem disappointing, it is actually encouraging to

suggest that moderate intensity exercise, both acute and longitudinal in nature, does not aggravate the chronically inflamed environment already observed in these patient populations. This is of particular importance in RTRs (Study 4 – Chapter 7), as there is a fine balance to be struck between immune system suppression and activation, and any significant immune system activation or perturbation of inflammatory state may represent an increased risk of graft loss (Dahle et al. 2011; Gurlek Demirci et al. 2015).

Chronically and systemically elevated levels of pro-inflammatory cytokines have been associated with worsened disease state, aortic stiffness, atherosclerosis, increased cardiovascular disease risk and increased all-cause and cardiovascular mortality in CKD and ESRD patients (Desjardins et al. 2017; Lee et al. 2015; Stenvinkel et al. 1999; Wanner et al. 2002). In Study 2, there was a reduction in the circulating concentration of TNF-α, despite roughly half of the samples displaying TNF-α values below the minimum detectable threshold of the technique. This is in line with previous research that has shown an anti-inflammatory effect of regular IDC training in HD patients (Peres et al. 2015; Wong et al. 2017). Whilst there are numerous possible mechanisms governing the anti-inflammatory effects of exercise in the general population (Gleeson et al. 2011), a potential cause of these reductions is alterations in circulating leukocyte phenotypes. In a recent study, Dungey et al found that 6-months of thrice-weekly moderate intensity IDC elicited a reduction in the circulating proportion of intermediate monocytes in the total monocyte pool (Dungey et al. 2017). Whilst leukocyte phenotypes were not analysed in Study 2 (primarily due to logistical constraints), a similar alteration would explain the observed cytokine changes as intermediate monocytes secrete a large proportion of pro-inflammatory mediators compared to their classical and non-classical counterparts (Merino et al. 2011). A possible mechanism behind the anti-inflammatory effect of exercise is a reduction in leukocyte activation. Previous research has shown that regular aerobic exercise training can downregulate monocyte and lymphocyte activation in pre-dialysis CKD patients (as measured by monocyte CD86 and HLA-DR expression and lymphocyte CD69 expression) (Viana et al. 2014). As explained in Chapter 2, chronic leukocyte activation as a result of reduced renal cytokine and uraemic toxin clearance is an integral factor in chronic systemic inflammation in CKD. As such, a reduction in markers of leukocyte activation would result in reduced

cytokine secretion by these cells and may exert an anti-inflammatory effect. However, leukocyte activation was not assessed in the studies included in this thesis – this warrant further investigation in the HD population.

It is unclear why the potential anti-inflammatory effect observed in Study 2 were not mimicked in Study 3 (Chapter 6), especially given the mean exercise session intensity in the IDC group was greater in Study 3. Although it may simply be a product of a shorter exercise intervention (12 weeks vs 24 weeks), effects were observed at 12 weeks in Study 2 and therefore this is unlikely. As mentioned previously, this may further point to the heterogeneity of the HD population. Whilst efforts can be made to create adequate exclusion criteria in order to generate more homogenous populations, the widely varying clinical characteristics (e.g. primary diagnosis, current comorbidities, HD vintage, current medications, fluctuations in treatment protocols, control of fluid status) of HD patients makes this difficult. Very large sample sizes would be necessary in order to stratify these variables and adjust for their impact on exercise adaption and, given the time and effort-intensive nature of exercise intervention delivery in HD patients, this is a significant undertaking. Additionally, it is perhaps disappointing that no positive effects of exercise were revealed in study 4 with regards to inflammation. However, as no negative inflammatory response was exhibited, regular moderate intensity aerobic exercise participation should be promoted to RTRs, and future research should investigate exercise regimes of different modalities and intensities.

Whilst between-group differences were observed in MIG and MCP-1 in Study 4 (elevated in the RTRs only), chemokines were unaffected by exercise in both Study 2 and Study 4 (chemokines were not analysed in Study 3 given the lack of changes in cytokine concentrations, the large inter-patient variability and the lack of changes in chemokine observed in Study 2). Whilst circulating pro- and anti-inflammatory cytokines are more commonly assessed in HD patients given their widespread influences as mentioned earlier, chemotaxis inducing cytokines (i.e. chemokines) represent an integral step in the pathway that links inflammation to atherosclerosis. Following adhesion to the vasculature, chemokines released by tissue-resident leukocytes (e.g. T cells and macrophages) drive the extravasation of their

target cell into the subendothelial space, ultimately resulting in endothelial dysfunction, vascular thickening and the initiation and progression of atherosclerotic plaque (Hansson 2003). Once resident within the vasculature, these leukocytes can secrete lytic enzymes (e.g. matrix-metalloproteinases) which can cause plaque destabilisation and increase the chance of plaque rupture and subsequent thrombotic events (Stenvinkel et al. 2005). Despite this, the current body of literature concerning chemokines in ESRD is limited and is non-existent when also concerning the impact of exercise. Therefore, further research is necessary to determine whether circulating chemokines are responsive to regular moderate intensity aerobic exercise in ESRD, as this may represent another avenue by which exercise can improve cardiovascular health in this population. As mentioned in Chapter 7, the elevations in MIG and MCP-1 in the RTRs in Study 4 may be an over-compensation response to the immunosuppressive regime. Whilst this could be considered alarming given that previous research has observed relationships between very high concentrations of these chemokines and allograft rejection, the elevations recorded here were markedly lower than those observed in the studies investigating this relationship.

Accordingly, a limitation of the studies included in this thesis is the absence of measures of adhesion molecules (e.g. VCAM-1, ICAM-1, E/P/L-Selectin) as these molecules govern the initial adherence of leukocytes to the endothelium before their subsequent extravasation. This is of particular importance for future research given that these molecules are elevated in ESRD patients and have been associated with systemic inflammation and vascular events (Papayianni et al. 2002).

8.3 Immune cell phenotypes

Whilst the concentration of circulating cytokines is largely a consequence of immune cell activity (non-leukocyte cells, e.g. endothelial cells, can also secrete cytokines), immune cell subsets and phenotypes were only directly measured in Study 4. This was primarily due to the importance of assessing these cells and how they respond to an acute bout of exercise in an immunosuppressed population. Encouragingly, the exercise elicited a reduction in the proportion of circulating intermediate ($CD14^{++}CD16^{+}$) monocytes within the total monocyte pool, which was similar across all three

populations (RTRs, non-dialysis-dependent CKD patients and healthy controls). This monocyte subset has been associated with elevated cardiovascular risk in CKD and ESRD patients (Heine et al. 2012) – this association is strengthened when this monocyte subset also displays a high degree of ACE expression (Ulrich et al. 2010). Previous research has investigated the response of these monocytes to acute (Van Craenenbroeck et al. 2014) and chronic (Dungey et al. 2017) aerobic exercise and observed a relative proportional increase and relative proportional decrease, respectively – this is a similar pattern to that observed in the general population (Simpson et al. 2009; Timmerman et al. 2008). However, this is the first study to investigate these relationships in RTRs and has revealed a different response pattern to acute exercise (i.e. a reduction in circulating proportion). Glucocorticoid treatment, which is often incorporated into the immunosuppressive regime of RTRs, can selectively deplete the intermediate monocyte subset (Fingerle-Rowson et al. 1998), and thus may be impacting the exercise-induced response. However, as this effect was also observed in the non-dialysis CKD and healthy control groups, the extent of the effect of glucocorticoid treatment in this case may be minimal. A reduction in the circulating proportion of intermediate monocytes is a previously reported mechanism by which exercise can exert an anti-inflammatory effect (Gleeson et al. 2011) and as such this is a promising finding which deserves further investigation, particularly with regards to chronic exercise training as it is yet to be determined if this effect persists or alters resting levels in this specific patient population.

Aside from the responses to exercise, there were significant between-group differences observed in leukocyte phenotypes. Whilst previous research has, to a limited extent, assessed leukocyte phenotypes in RTRs and demonstrated a general lymphopenia (Hutchinson et al. 2003), this is, to the author's knowledge, the first study to quantify T-regs in this population. This is unusual, given the importance of these cells in immune regulation and maintenance of graft tolerance (Wood and Sakaguchi 2003). However, as they were reduced in both RTR and CKD populations, it seems to be a response to reduced renal function *per se* rather than the immunosuppressive regime. As with the inflammatory response, the lack of exercise-induced response in the number of these cells suggests that this exercise modality is immunologically safe for RTRs. However, the relative proportion of T-regs increased in response to exercise in all groups, suggesting a possible anti-inflammatory and thus beneficial effect which may

contribute to graft tolerance in the RTRs. Lastly, as explained in Chapter 7, the monocyte ACE expression results are interesting, but all theories currently remain speculative. It is as yet unknown whether the number of ACE^+ monocytes or the degree of monocyte ACE expression is more clinically relevant with regards to vasoconstriction. As monocyte ACE expression has been associated with increased cardiovascular disease prevalence and all-cause mortality in ESRD patients (Ulrich et al. 2006, 2010), this question warrants further investigation in the RTR population.

8.4 Microparticles

The studies included in this thesis aimed to characterise the MP response to acute aerobic exercise in the general population (Study 1), then investigate this response to longitudinal aerobic exercise training in HD patients (Study 2) and then finally to examine the response to acute aerobic exercise in RTRs (Study 4).

MPs have been found to exert a number of pathophysiological effects. MPs from a variety of cellular sources can act on the endothelium to reduce NO bioavailability and promote endothelial dysfunction, induce endothelial expression of adhesion molecules (VCAM-1, E-Selectin), thus promoting cellular adhesion and driving atherosclerosis, and induce endothelial cells and leukocytes to release pro-inflammatory cytokines and chemokines (IL-6, IL-8 and RANTES) (Lovren and Verma 2013). Additionally, the phosphatidylserine and TF externalised during MP formation can provide a platform for platelet-mediated coagulation and thrombosis (Del Conde et al. 2005; Polgar et al. 2005). As such, a reduction in the circulating MP count and/or a reduction in MP TF expression would be expected to represent a reduction in these pathophysiological processes, and this is principally what was observed in these studies in response to exercise. Study 1 (Chapter 4) revealed a reduction in the proportion of platelet and neutrophil-derived MPs that were TF-positive after acute exercise in a group of healthy individuals, and Study 4 (Chapter 7) displayed a reduction in TF expression on platelet-derived MPs after acute exercise in healthy individuals, RTRs and CKD patients. Therefore, it seems that acute aerobic exercise can have a positive influence on MP characteristics in both CKD and non-CKD populations.

However, the opposing argument to the beneficial effects of reducing MP counts is two-fold. Firstly, little is known about the mechanism by which MPs are cleared from the circulation. Previous research has suggested that platelet-derived MPs are opsonised by the complement system and subsequently engulfed and dismantled by phagocytes (Flaumenhaft 2006), whilst non-opsonised MPs can be removed by antibody-mediated (IgM) macrophage clearance (Litvack, Post, and Palaniyar 2011). However, other results have shown that MPs, particularly those formed under increased shear stress conditions, can adhere to the endothelium and thus increase endothelial activation and drive adhesion molecule expression (Nomura et al. 2001). Therefore, it may be that the mechanism or cause of MP clearance from the circulation determines the nature of the effect of MP reduction (i.e. an inert and deliberate clearance mechanism vs a biologically active and pathophysiological sequestration). Secondly, there is a small but growing body of research outlining the potential beneficial effects of circulating MPs (Highton et al. 2018). Firstly, the responses that can become chronically overstimulated and dysregulated (i.e. accelerated coagulation and thrombosis) are a necessary response to vascular injury when appropriately regulated. Additionally, MPs can deliver RNAs growth factors and ligate cell surface receptors on target cells and thus facilitate inter-cellular communication (Ferraris 2015). Lastly, MPs can prevent the premature apoptosis of leukocytes and endothelial cells, potentially mediated by increased TGF-β1 and decreased caspase 3 levels (Brunetti et al. 2000; Hussein et al. 2007).

In summary, whilst the reduction in MP count and TF expression in response to acute exercise can be primarily considered a positive response and warrant further investigation, little is known about the clearance mechanism of these MPs. Additionally, the possible beneficial effects of MPs deserve consideration. Given the unspecific and varying causes of MP formation, the widespread mechanistic actions of MPs and the lack of uniformity of analysis techniques currently present in the literature (Highton et al. 2018), there is a great deal more research to be done in this complex but promising field.

8.5 Feasibility of intradialytic exercise

Despite the lack of improvement in physical performance in Study 2 and the lack of changes in circulating cytokine concentrations in Study 3, intradialytic exercise seems to be a feasible and accepted

exercise modality. As explained in Chapters 5 and 6, whilst interdialytic exercise programmes may be more beneficial in terms of exercise adaption, compliance rates are low and dropout rates are high. This is most likely due to the time commitment necessary for these programmes, as they require additional time beyond that already being given to dialysis treatment. Intradialytic exercise demands no additional time commitment and is also completed under the supervision of exercise professionals in a healthcare environment, alleviating possible safety concerns. Therefore, it appears the most ideal exercise intervention is the one the patients can regularly complete, rather than that which provides the most adaptation benefits when (and if) completed. This point was supported by the studies included here, with compliance rates of 82% (Study 2) and 93% (Study 3) for IDC, and 91% for LF-EMS (Study 3). The results regarding LF-EMS treatment are particularly encouraging given the demonstrated improvements in performance. Consequentially, further research should be completed that investigates the practicality and efficacy of this modality in patients with functional limitations who are incapable of completing more traditional exercise modalities.

However, there are several problems inherent with intradialytic exercise that must be addressed. Firstly, the studies completed here were completed by researchers dedicated to the provision of intradialytic exercise to the patients with specifically designed equipment. For this exercise modality to transition out of research and into a practical setting, either exercise professionals must be employed, or HD nurses must be trained to prescribe and deliver exercise programmes, adding to their current workload. Additionally, the necessary equipment (either the specially designed cycle ergometers or the LF-EMS apparatus) must be provided. When considering these factors, less equipment and staff-dependent exercise regimes may be considered more desirable, for instance at-home walking programmes (Viana et al. 2014). Secondly, there is a need to individualise exercise prescription in this patient population. Recent research has suggested that the proportion of clinically significant responders to intradialytic exercise training is low (<50%) (Valenzuela et al. 2018) and as such individualisation is needed. Similarly, as described in Chapter 5, there may be a certain proportion of the HD population that do not find IDC to be an acceptable exercise modality, and this may have inserted a degree of bias into the presented results. Given the large number of variables that may impact exercise

adaptation (e.g. HD vintage, age, physical activity levels, comorbidities, primary diagnosis etc.), large-scale studies are necessary to stratify patients based on these characteristics before guidelines on individualisation can be created. In a practical setting, this will help to increase the efficacy of IDC in this population, as it will help to identify those patients who are most at need and most likely to benefit from targeted exercise interventions, increasing the overall effectiveness.

8.6 Potential applications and future research

Whilst some of the exercise modalities featured in the studies included in this thesis failed to elicit the expected beneficial effects (e.g. no change in physical function in Study 2, no change in circulating cytokine concentration in Study 3), it is encouraging to note that inflammation and immune function were not negatively impacted. Therefore, the results presented here add to the current evidence base supporting the safety of moderate intensity aerobic exercise in these patient groups.

This outcome has two important implications. Firstly, IDC is well tolerated and immunologically safe, and therefore should be recommended and provided as part of regular HD treatment where possible. Whilst concerns have been raised over blood pressure variations during HD in combination with IDC, post-acute IDC hypotension has been shown to be asymptomatic and represents a normal blood pressure response to exercise (Dungey et al. 2015). Therefore, from a physiological perspective, there is no reason not to recommend this type of exercise training in HD patients, particularly when those who are less physically capable can complete LF-EMS training as an effective substitute. However, further research should aim to quantify a larger battery of pro- and anti-inflammatory cytokines on a larger cohort. Similarly, the cost-effectiveness of this type of training must be investigated – i.e. the cost of implementing the exercise programme versus the potential healthcare savings elicited by a more physically active patient population. Additionally, the feasibility of LF-EMS training in more functionally limited patients must be investigated.

Secondly, these results also point towards the safety of moderate intensity aerobic exercise participation in RTRs. As no exacerbation of inflammatory status or immune cell distribution was observed, this exercise modality can be safely recommended in this patient population. Further

research should focus on two areas: investigating the impact of a longitudinal exercise training intervention on these parameters; and investigating different exercise intensities (e.g. high-intensity interval training) and modalities (e.g. cycling) to further inform exercise guidelines for RTRs recipients and allow them to reap the same benefits of regular exercise participation as the general population.

Lastly, an overarching theme that has been identified in the studies presented here is that, for the most part, the sample sizes included were not large enough to detect significant changes in the inflammatory and immune measures in response to the interventions employed. Future research studies should identify key markers and employ *a priori* sample size calculations to ensure that they are adequately powered to detect changes in the marker of interest. In this thesis, whilst a sample size calculation was completed for Study 4, Study 1 and Study 3 were completed using secondary analysis of data from other studies, and Study 2 used a selected cohort (that was a large as possible from a laboratory analysis feasibility prospective) from a larger study, and as such *a priori* sample size calculations were not completed.

8.7 Conclusions

In summary, moderate intensity aerobic exercise can positively impact circulating MP phenotypes in both the general and ESRD populations. Additionally, whilst regular IDC may promote an anti-inflammatory environment in HD patients, it most certainly does not elicit a detrimental response, and acute moderate intensity aerobic exercise can elicit a favourable shift in immune cell subsets in RTRs. These results add to the evidence base that demonstrates the safety of aerobic exercise in these patient populations, and as such regular participation should be encouraged. Further work should be completed to inform exercise prescription guidelines in the end-stage renal disease population.

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Appendices

Appendix 1 - Microparticle Analysis Methodology Development – Centrifugation Protocol

Aim

To investigate the effects of the centrifugation protocol used to generate platelet-free (or 'platelet-poor') plasma (PFP). The two-step centrifugation procedure routinely used to generate PFP should maximise the recovery of MPs from venous blood samples by removing all unnecessary (and larger) objects from the sample. The first centrifugation should ensure the removal of cells (i.e. erythrocytes and leukocytes) whilst the second centrifugation should ensure the removal of platelets.

Methods

The centrifugation protocol to be assessed was as follows:

1st spin: 2500g, 15 mins, 20°C – followed by removal and aliquotting of supernatant

2nd spin: 2500g, 15 mins, 20°C – followed by removal and storage of supernatant (intended for future MP analysis)

This protocol was carried out on 5 venous blood samples, collected into sodium citrate tubes from patients during their sample collection protocols in their respective studies (CYLCE-HD and PINK).

Aliquots of the following were sequentially set aside during the centrifugation protocol:

- Whole blood (before any centrifugation) = **A1**
- The supernatant created by the first centrifugation. This should be platelet-rich but with the majority of the erythrocytes and leukocytes removed = **A2**
- The pellet created by the first centrifugation. This should contain the majority of the cells removed from A2 during centrifugation, and platelets = **A3**
- The supernatant created by the second centrifugation. This should be PFP, containing few cells or platelets and thus be suitable for MP analysis = **A4**
- The pellet created by the second centrifugation. This should contain the majority of the platelets removed from A4 during centrifugation = **A5**

These 5 aliquots were analysed using an automated cell counter (Yumizen H500, Horiba, Northampton UK). This automated analyser generated results for the concentrations of: red blood cells; platelets; total white blood cells; neutrophils; lymphocytes; monocytes; eosinophils, and basophils.

Results

The mean concentrations of each cell type in each aliquot are presented in Table 1.

	RBC ($x10^{12}$/L)	**PLT ($x10^9$/L)**	**WBC ($x10^9$/L)**	**NEU ($x10^9$/L)**	**LYM ($x10^9$/L)**	**MON ($x10^9$/L)**	**EOS ($x10^9$/L)**	**BAS ($x10^9$/L)**
A1	4.71 (0.946)	212.0 (103.1)	5.784 (1.889)	3.484 (1.791)	1.514 (0.328)	0.508 (0.185)	0.186 (0.104)	0.068 (0.013)
A2	0.002 (0.004)	4.4 (1.9)	0.060 (0.021)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
A3	9.346 (2.365)	255.0 (275.7)	5.230 (5.856)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
A4	0.008 (0.004)	4.4 (2.2)	0.052 (0.023)	0.010 (0.022)	0.006 (0.013)	0.002 (0.004)	0.006 (0.013)	0.000 (0.000)
A5	0.004 (0.005)	9.0 (1.9)	0.054 (0.011)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)

Table 1. Mean data for each cell type and each aliquot. RBC = Red blood cell. PLT = Platelet. WBC = White blood cell. NEU = Neutrophil. LYM = Lymphocyte. MON = Monocyte. EOS = Eosinophil. BAS = Basophil. Data are presented as 'mean (SD)'.

As can be seen in Table 1 and Figure 1A-C, the two step centrifugation protocol was successful in depleting the samples of red/white bloods cells and platelets, creating an acellular and platelet-free sample (A4).

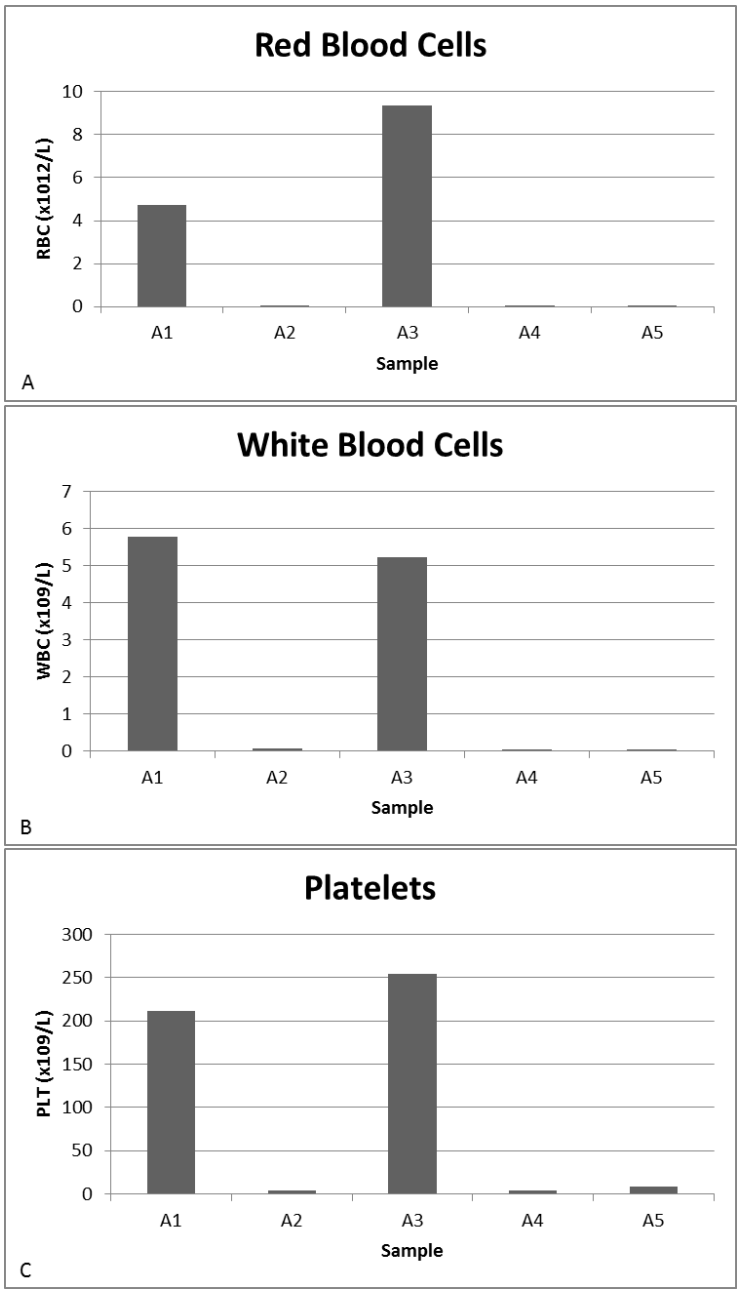


Figure 1. A graphical demonstration of the number of RBCs (A), WBCs (B) and platelets (C) present in each sample throughout the centrifugation procedure.

Conclusion

The sample designated for MP analysis (i.e. A4, the aliquoted supernatant removed following the second spin) was rendered largely acellular and platelet free by the two step centrifugation protocol. As such, it is suitable to use for the analysis of MPs.

Appendix 2 – Data and calculations for coefficient of variation measures

The data that was used for the calculation of coefficient of variation in the laboratory techniques (cytometric bead array and flow cytometric analysis of microparticles) is included in the table below. In both cases, repeatability analysis was completed on a cohort of the samples included in this thesis, rather than on the whole dataset. This was primarily due to the cost and time-intensive nature of completing flow cytometry. Coefficient of variation values were calculated using SPSS (IBM SPSS v. 24) for each duplicate/repeated analysis and then averaged (mean) across each technique

Cytometric bead array

After the removal of samples that fell below the minimum detectable threshold (0.274 pg/mL – primary results pertaining to IL-2 and IL-17a) intra-assay (duplicate samples analysed within same test) repeatability analysis was completed on 61 samples. Raw data is provided as fg/mL. This resulted in a mean coefficient of variation of 10.08%.

Flow cytometric analysis of microparticles

Intra-assay (duplicate samples analysed within same test) repeatability analysis was completed on 72 samples. Raw data is reported as number of events pertaining to the number of events positively detected as expressing a cell-specific marker (i.e. CD42b, CD66b, CD144 or CD14). This data is reported after deducting the number of events identified using the control markers to account for non-specific staining and auto-fluorescence. This resulted in a mean coefficient of variation of 13.88%.

Cyt_1	Cyt_2	Cyt_CV	Cyt_CV (%)	MP_1	MP_2	MP_CV	MP_CV (%)
922.8	1000.48	0.0571	5.71	127	147	0.1032	10.32
330.52	382.43	0.103	10.3	104	138	0.1987	19.87
5897.55	5350.43	0.0688	6.88	129	148	0.097	9.7
8646.05	7994.31	0.0554	5.54	123	149	0.1352	13.52
5181.4	5792.35	0.0787	7.87	132	149	0.0856	8.56
9041.88	9887.2	0.0632	6.32	223	259	0.1056	10.56
8099.06	8961.31	0.0715	7.15	176	148	0.1222	12.22
4491.63	4989.8	0.0743	7.43	54	86	0.3232	32.32
13815.51	12724.54	0.0581	5.81	108	133	0.1467	14.67
16942.78	15856.26	0.0468	4.68	57	72	0.1644	16.44
6296.97	6003.91	0.0337	3.37	20	38	0.4389	43.89
7552.48	6983.91	0.0553	5.53	34	39	0.0969	9.69
7390.97	7026.2	0.0358	3.58	40	49	0.143	14.3
7199.06	7912.95	0.0668	6.68	40	31	0.1793	17.93
6463.43	4923.6	0.1912	19.12	28	33	0.1159	11.59
2094.18	2455.27	0.1122	11.22	32	28	0.0943	9.43
42107.52	38885.93	0.0563	5.63	52	43	0.134	13.4
2346.12	2193.63	0.0475	4.75	34	41	0.132	13.2
9463.75	8861.48	0.0465	4.65	50	58	0.1048	10.48
6806.36	6623.24	0.0193	1.93	77	95	0.148	14.8
9999.44	9688.54	0.0223	2.23	79	93	0.1151	11.51
8264.77	8969.81	0.0579	5.79	96	77	0.1553	15.53
18944.92	16653.29	0.091	9.1	85	67	0.1675	16.75
2568.38	2237.44	0.0974	9.74	182	211	0.1044	10.44
11855.75	10595.64	0.0794	7.94	81	71	0.093	9.3
3019.08	2659.44	0.0896	8.96	29	31	0.0471	4.71
1520.49	700.88	0.5218	52.18	58	70	0.1326	13.26
683.53	2591.51	0.8239	82.39	48	61	0.1687	16.87
401.24	288.35	0.2315	23.15	17	25	0.2694	26.94
1922.71	2097.08	0.0613	6.13	28	16	0.3857	38.57
920.12	801.53	0.0974	9.74	9	10	0.0744	7.44
4285.38	4067.78	0.0368	3.68	16	20	0.1571	15.71
1463.14	1202.4	0.1383	13.83	11	18	0.3414	34.14
1416.86	1309.92	0.0555	5.55	19	24	0.1644	16.44
514.57	487.29	0.0385	3.85	21	29	0.2263	22.63
922.16	814.4	0.0878	8.78	22	28	0.1697	16.97
1347.08	1262.38	0.0459	4.59	70	79	0.0854	8.54
289.18	360.85	0.1559	15.59	109	133	0.1403	14.03
1258	1160.6	0.057	5.7	109	137	0.161	16.1
9494.85	9113.03	0.029	2.9	121	107	0.0868	8.68
860.02	780.01	0.069	6.9	118	129	0.063	6.3
1600.12	1499.85	0.0457	4.57	242	220	0.0673	6.73
344.77	353.28	0.0173	1.73	173	198	0.0953	9.53
707.84	606.31	0.1093	10.93	66	74	0.0808	8.08
2946.25	2332.28	0.1645	16.45	138	115	0.1286	12.86
1067.43	841.55	0.1673	16.73	30	26	0.101	10.1
4126.15	3835.58	0.0516	5.16	27	24	0.0832	8.32
1607.09	1851.74	0.1	10	23	24	0.0301	3.01
571.76	508.76	0.0825	8.25	28	23	0.1386	13.86

892.26	658.53	0.2132	21.32	22	27	0.1443	14.43
1220.04	1555.14	0.1708	17.08	23	29	0.1632	16.32
1099.25	1201.36	0.0628	6.28	29	26	0.0771	7.71
1021.94	853.35	0.1271	12.71	20	35	0.3857	38.57
1099.25	1185.05	0.0531	5.31	20	15	0.202	20.2
4928.5	4580.02	0.0518	5.18	43	49	0.0922	9.22
1269.21	1101.36	0.1001	10.01	62	76	0.1435	14.35
737.41	697.76	0.0391	3.91	99	92	0.0518	5.18
388.02	430.06	0.0727	7.27	70	83	0.1202	12.02
380.1	437.01	0.0985	9.85	75	94	0.159	15.9
1809.72	1475.04	0.1441	14.41	176	172	0.0163	1.63
645.13	690.49	0.048	4.8	86	95	0.0703	7.03
				33	37	0.0808	8.08
				52	59	0.0892	8.92
				14	17	0.1369	13.69
				11	11	0	0
				15	12	0.1571	15.71
				9	8	0.0832	8.32
				21	27	0.1768	17.68
				11	13	0.1179	11.79
				9	11	0.1414	14.14
				30	24	0.1571	15.71
				12	10	0.1286	12.86
Mean CV (%)			**10.08**				**13.88**

Appendix 3 – MP analysis protocol

Phenotypic flow cytometric analysis of microparticles

Date:	
Samples Analysed:	

BUFFERS
All buffers are made as 10X stock

For 1X solution:

- Prepare on the day of use
- Dilute 1 in 10 with 0.2µm filtered water (e.g. 2ml in 18ml)
- Filter again with 0.2µm syringe filters
- Store all solutions at room temperature

Recipes for 10X stock solutions:

MP buffer	*10X conc.*	*mwt*	*Weight for 100ml*	*Final conc.*
NaCl	1.45M	58.44	8.47g	145mM
KCl	27mM	74.55	0.20g	2.7mM
HEPES	0.1M	238.30	2.38g	10mM

An Va buffer	*10X conc.*	*mwt*	*Weight for 100ml*	*Final conc.*
NaCl	1.4M	58.44	8.18g	145mM
$CaCl_2$	25mM	110.98	0.28g	2.5mM
HEPES	0.1M	238.30	2.38g	10mM

An Vb buffer	*10X conc.*	*mwt*	*Weight for 100ml*	*Final conc.*
NaCl	1.4M	58.44	8.18g	140mM
HEPES	0.1M	238.30	2.38g	10mM

ANTIBODIES USED

Antibody	Antigen	Flourochrome	Isotype	BD Code	Dilution	Dilution*
AnV	AnV	FITC		556420	Neat	
IgG1k	Control IgG1k	PE APC	IgG1k	559320	1 in 2	**10 + 10** **10 + 10**
CD42b	Platelet	PE	IgG1k	555473	Neat	
CD144	Endothelial cell	PE	IgG1k	560410	Neat	
CD142	Tissue Factor	APC	IgG1k	365206**	Neat	
IgMk	Control IgMk	PE	IgMk	555584	1 in 16	**2 + 30**
CD66b	PMN	PE	IgMk	561650	Neat	
IgG2ak	Control IgG2ak	PE	IgG2ak	555574	1 in 16	**2 + 30**
CD14	Monocytes	PE	IgG2ak	555398	Neat	

* = enough for **one sample**, volume of antibody + volume of An Va buffer, both in µl
** = CD142 is from Biolegend and must be ordered separately

Procedure	*Time*
PFP samples out of -80ºC freezer	
Finish MP washing	
Finish MP staining	
Start acquisition on flow cytometer	
Time delay from staining to acquisition	

PFP SAMPLE PREPARATION

1. Take 4.3ml whole blood onto sodium citrate (minimum 21 gauge needle)
2. Spin at 2,500g for 15 mins at 18-20ºC
3. Take the top 2ml of plasma and aliquot as 2x 1ml in Eppendorfs (Platelet Rich Plasms)
4. Spin at 2,500g for 15 mins at 18-20ºC
5. Gently pipette the top 750µl into 6x 250µl (Platelet Free Plasma)
6. Store in labelled Eppendorfs at -80ºC
7. If required, also store 250µl aliquots of PRP as controls for MP gating

MP WASHING METHOD

1. Thaw PFP samples at room temperature
2. Prepare and filter MP, AnVa and AnVb buffers
3. Spin at 18,000g at room temperature for 30 mins (hinge on the outside)
4. Very gently remove 90% (225µl) of the supernatant volume, with the hinge pointing upwards
5. Very gently, drop by drop, add 90% volume (225µl) MP buffer
6. Spin at 18,000g at room temperature for 30 mins (hinge on the outside)
7. Very gently remove 90% (225µl) of the supernatant volume, with the hinge pointing upwards
8. Very gently, drop by drop, add 90% volume (225µl) MP buffer
9. Mix very gently by 4x pipetting

MP STAINING METHOD

1. Dilute isotype control antibodies
2. Use all phenotyping antibodies neat
3. Label FACS tubes as detailed below
4. Pipette MP, buffer and antibody into tubes as detailed below
5. Gently flick the base of the tube to mix
6. Incubate in the dark at room temperature for 25 minutes
7. Dilute samples in the appropriate buffer for analysis (grey columns below)

SAMPLE TUBES

			Incubate for 25' RT dark					Add after 25'	
Tube		***Stain***	***MP***	***AnV***	***Ab***	***AnVa***	***AnVb***	***AnVa***	***AnVb***
1	Control	JMP	10µl			10 µl		310µl	
2	AnV control	AnV control	10µl	1.5µl			8.5µl		300µl
3	control	AnV IgG1k-PE IgG1k-APC	10µl	1.5µl	 5µl 5µl	8.5µl		300µl	
4	control	AnV IgM-PE IgG1k-APC	10µl	1.5µl	 5µl 5µl	8.5µl		300µl	
5	control	AnV IgG2a-PE IgG1k-APC	10µl	1.5µl	 5µl 5µl	8.5µl		300µl	
6	PLT TF AnV	CD42b-PE CD142-APC	10µl	 1.5µl	5µl 5µl	3.5µl		305µl	
7	PMN TF AnV	CD66b-PE CD142-APC	10µl	 1.5µl	5µl 5µl	3.5µl		305µl	
8	Mo TF AnV	CD14-PE CD142-APC	10µl	 1.5µl	5µl 5µl	3.5µl		305µl	
9	EC TF AnV	CD144-PE CD142-APC	10µl	 1.5µl	5µl 5µl	3.5µl		305µl	

MP ACQUISITION METHOD

1. All parameters must be H (e.g. SSC-H)
2. All parameters must be log (axes will look unusual if they are linear)
3. SSC-H threshold set at 10,000
4. Apply parameters to all samples
5. Flow rate must be SLOW
6. Acquire each sample for 2 mins
7. Once finished, export all data

Appendix 4 – MP analysis antibody volume optimisation

Aim

This experiment is designed to determine the optimal amount of antibody for the MP samples. This is the lowest amount that elicits maximal (or near maximal) mean fluorescence intensity (MFI). Once the MFI stops increasing with increasing Ab volume, the optimal volume has been reached. The optimal antibody volume for all phenotypes will be that which consistently produces the largest MFI across the most phenotypes (not necessarily all of the phenotypes).

Method

Antibodies for the MP phenotypes will be incubated with Platelet-Free-Plasma at increasing volumes, as shown in Table 1. To ensure the total volume in each tube remains consistent, the volume of the inert buffer (Buffer A) will be progressively decreased accordingly. These samples will be then be routinely acquired on an Accuri C6 Flow Cytometer (medium flow rate for 2 minutes) to produce MFI results for each sample tube.

The antibodies used for each MP phenotype are as follows:
Tissue Factor = CD142
Platelet = CD42b
Polymorphonuclear Neutrophil = CD66b
Monocyte = CD14
Endothelial Cell = CD144

Volumes and tube setup:

10µl MP sample in all tubes

Buffer A	**16.5µl**	**14.5µl**	**11.5µl**	**6.5µl**
Antibody	**0µl**	**2µl**	**5µl**	**10µl**
TF CD142 (FL-4) Plot 13	**1**	**2**	**3**	**4**
Plt CD42b (FL-2) Plot 14	**5**	**6**	**7**	**8**
PMN CD66b (FL-2) Plot 14	**9**	**10**	**11**	**12**
Mon CD14 (FL-2) Plot 14	**13**	**14**	**15**	**16**
EC CD144 (FL-2) Plot 14	**17**	**18**	**19**	**20**

Table 1. Antibody and buffer volumes for each tube

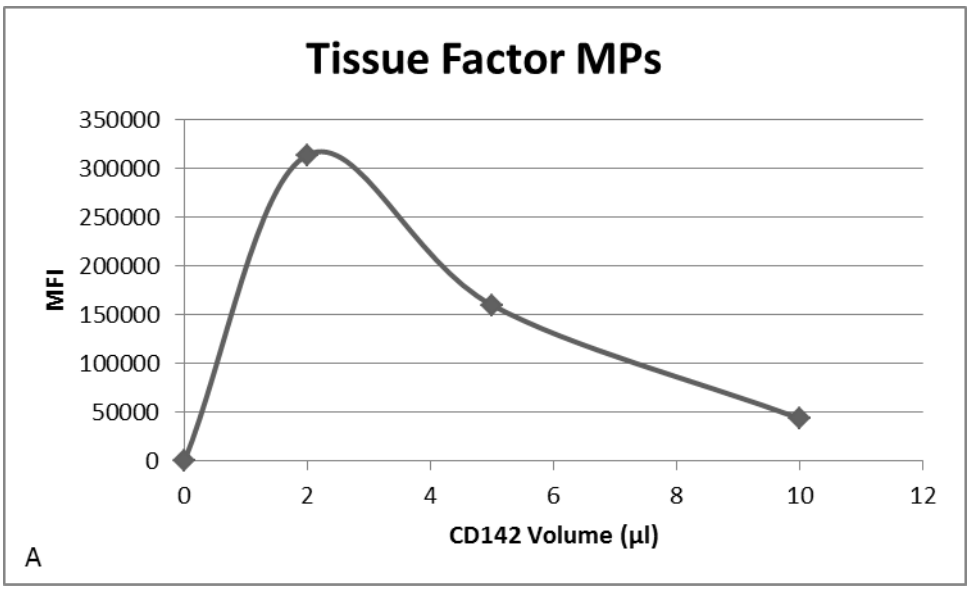
Incubate at room temp for 25 mins, then add 300µl buffer A and acquire.

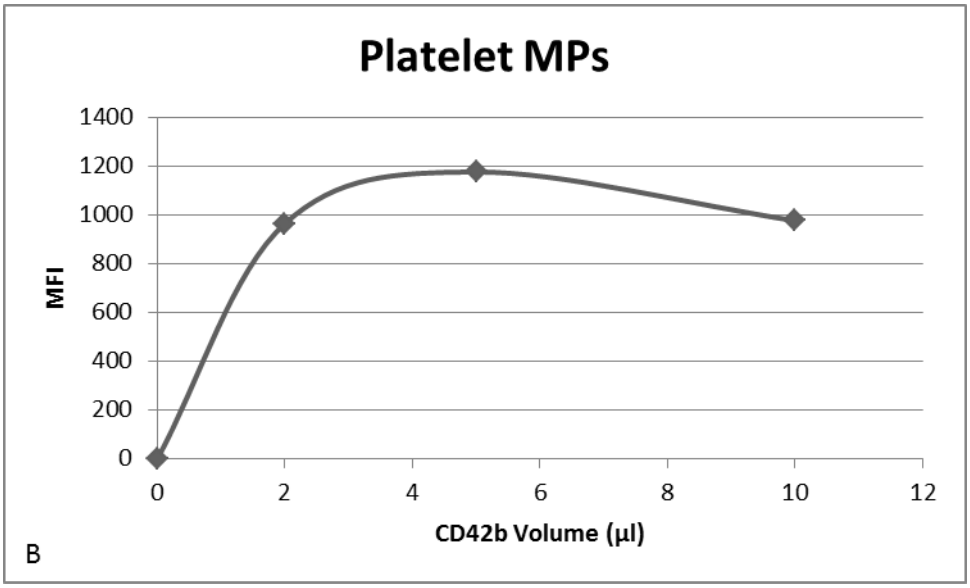
Results

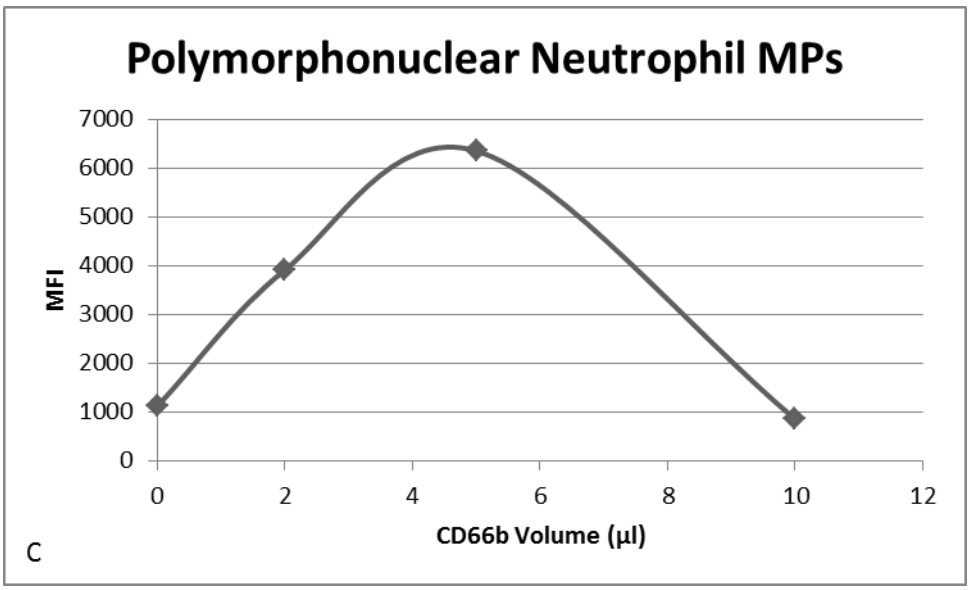
The MFI values for each tube are presented in table 2, and graphically in Figure 1A-E.

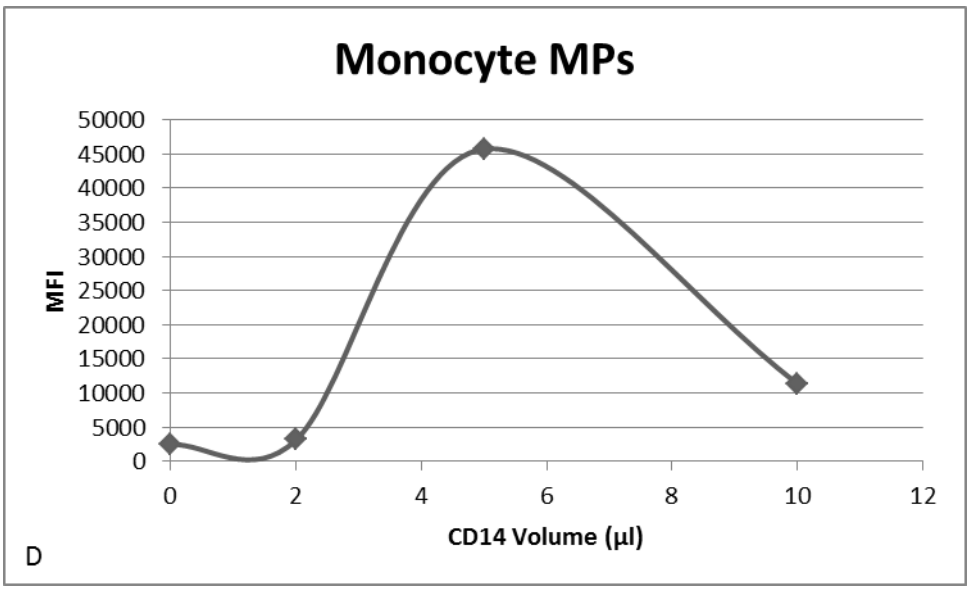
TF CD142 (FL-4)	**1 = 0**	**2 = 313951**	**3 = 159863**	**4 = 43559**
Plt CD42b (FL-2)	**5 = 0**	**6 = 966**	**7 = 1178**	**8 = 978**
PMN CD66b (FL-2)	**9 = 1126**	**10 = 3922**	**11 = 6363**	**12 = 860**
Mon CD14 (FL-2)	**13 = 2463**	**14 = 3137**	**15 = 45739**	**16 = 11357**
EC CD144 (FL-2)	**17 = 6512**	**18 = 17440**	**19 = 51145**	**20 = 399690**

Table 2. MFI values for each phenotype with increasing antibody volume.


Tissue Factor MPs
350000
300000
250000
200000
150000
100000
50000
0
MFI
0
2
4
6
8
10
12
CD142 Volume (µl)
A


Platelet MPs
1400
1200
1000
800
600
400
200
0
MFI
0
2
4
6
8
10
12
CD42b Volume (µl)
B


Polymorphonuclear Neutrophil MPs
7000
6000
5000
4000
3000
2000
1000
0
MFI
0
2
4
6
8
10
12
CD66b Volume (µl)
C



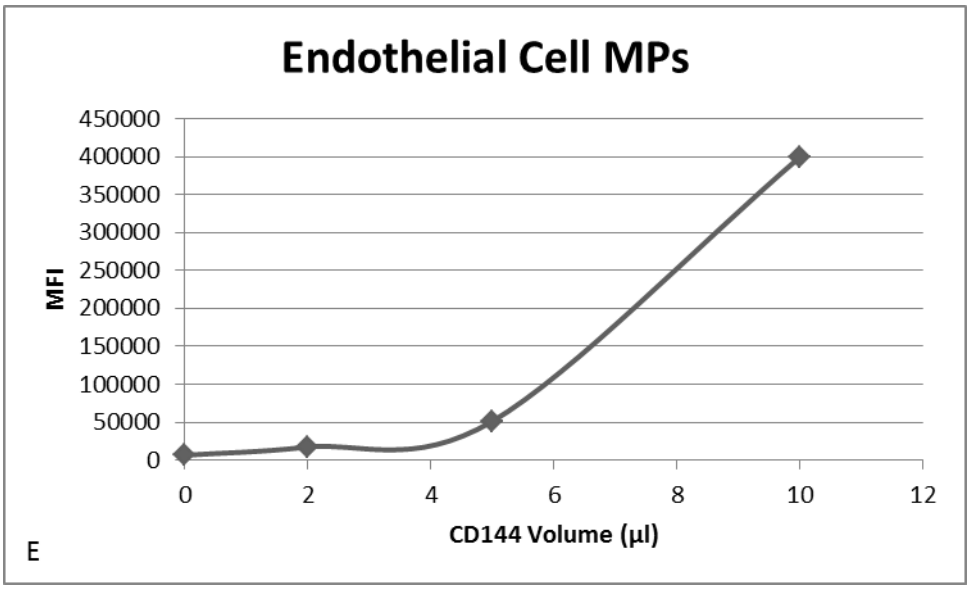


Figure 1. MFI values for increasing antibody volumes for each phenotype; Tissue Factor-positive MPs (A), Platelet MPs (B), Neutrophil MPs (C), Monocyte MPs (D) and Endothelial Cell MPs (E).

Optimal volumes for each antibody:

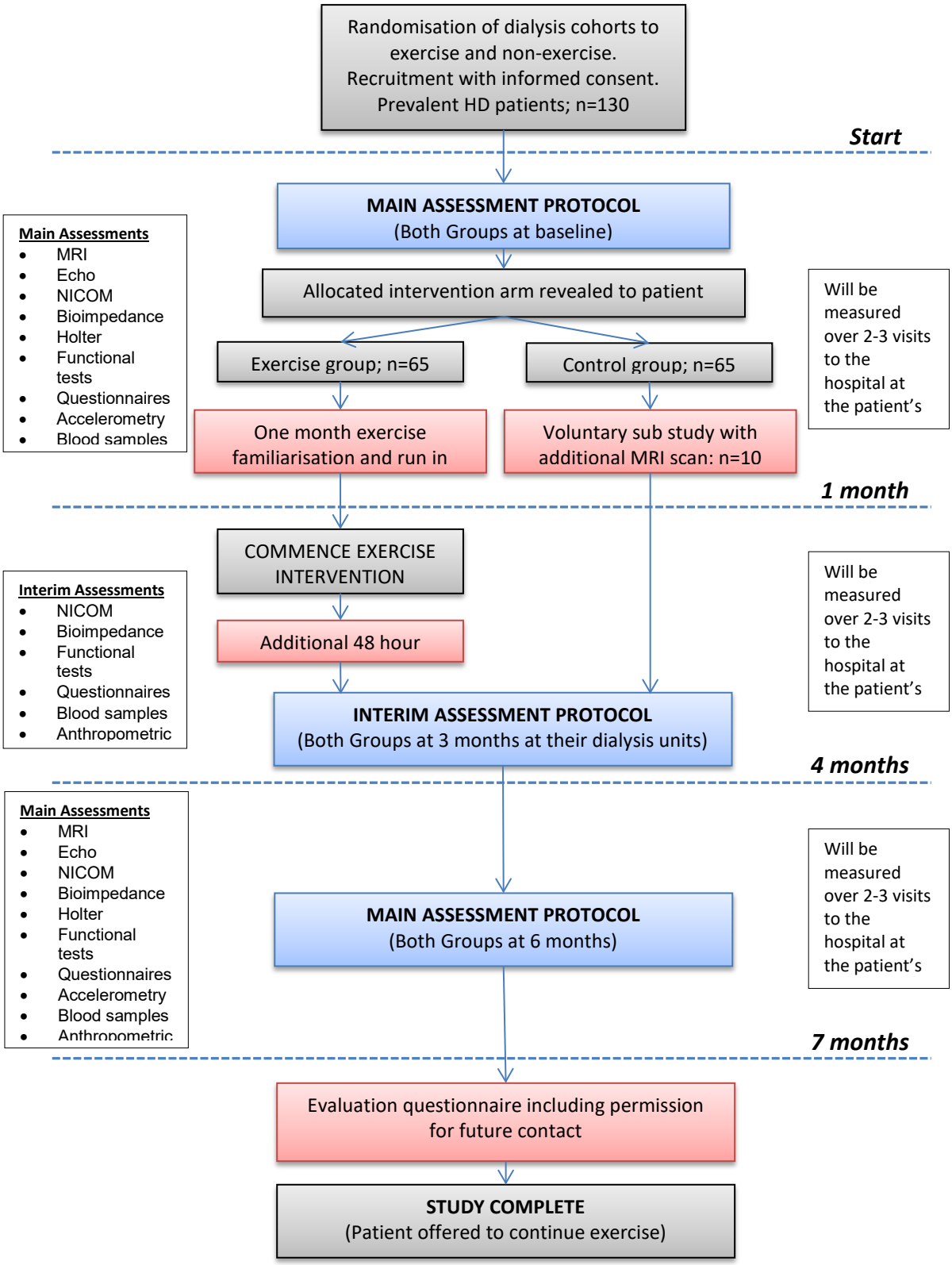
- TF CD142 = 2µl
- Plt CD42b = 5µl
- PMN CD66b = 5µl
- Mon CD14 = 5µl
- EC CD144 = 10µl

Conclusions

As 5µl is the most frequently optimal antibody volume for MP characterisation, it will be used across all phenotypes to ensure consistency of both available antibody binding, total tube volume and

antibody:sample ratio. Therefore, 5µl will be used for all phenotypes for subsequent MP analysis by flow cytometry.

Appendix 5 – CYCLE-HD protocol outlining study timeline and included assessments



Appendix 6 – Copy of the Leisure Time Exercise Questionnaire

LEISURE TIME EXERCISE QUESTIONNAIRE

1. If you currently exercise, how many times in the last 7 days have you done the following type of exercise?

   a) **Strenuous exercise** (Heart beats rapidly, i.e. running, jogging, squash, basketball, vigorous swimming, long distance bicycling)

   Number of times_____

   b) **Moderate exercise** (Not exhausting, i.e. fast walking, tennis, easy bicycling, volleyball, badminton, easy swimming)

   Number of times_____

   c) **Mild exercise** (Minimal effort, i.e. fishing, golf, easy walking)

   Number of times_____

2. In a 7 day period how often do you engage in any regular activity long enough to **work up a sweat** (heart beats rapidly)

   Often ☐ Sometimes ☐ Never/Rarely ☐

3. What activities do you usually do when you exercise?

Walking	☐	Cycling	☐	Running ☐
Swimming	☐	Weightlifting	☐	Aerobics ☐
Basketball	☐	Football	☐	Badminton ☐
Table Tennis	☐	Tennis	☐	Hiking ☐
Others (*please state*)	☐	_____		

Thank you for completing this questionnaire