Effect of high intensity interval training and moderate intensity continuous training on lymphoid, myeloid and inflammatory cells in kidney transplant recipients.

Ganisha M Hutchinson[1], Andrea M Cooper[1] Roseanne E Billany[3], Daniel G D Nixon[2], Nicolette C Bishop[3], Alice C Smith[2]

[1] Department of Respiratory Sciences, University of Leicester, UK
[2] Department of Health Sciences, University of Leicester, UK
[3] School of Sport, Exercise and Health Sciences, Loughborough University, UK

ABSTRACT

Kidney transplantations are seen to be a double-edge sword. Transplantations help to partially restore renal function, however there are a number of health-related co-morbidities associated with transplantation. Cardiovascular disease (CVD), malignancy and infections all limit patient and graft survival. Immunosuppressive medications alter innate and adaptive immunity and can result in immune dysfunction. Over suppression of the immune system can result in infections whereas under suppression can result in graft rejection. Exercise is a known therapeutic intervention with many physiological benefits. Its effects on immune function are not well characterised and may include both positive and negative influences depending on the type, intensity, and duration of the exercise bout. High intensity interval training (HIIT) has become more popular due to it resulting in improvements to traditional and inflammatory markers of cardiovascular (CV) risk in clinical and non-clinical populations. Though these improvements are similar to those seen with moderate intensity exercise, HIIT requires a shorter overall time commitment, whilst improvements can also be seen even with a reduced exercise volume.

The purpose of this study was to explore the physiological and immunological impact of 8-weeks of HIIT and moderate intensity continuous training (MICT) in kidney transplant recipients (KTRs). In addition, the natural variations of immune and inflammatory cells in KTRs and Non-chronic kidney disease (non-CKD) controls over a longitudinal period are explored. Newly developed multi-colour flow cytometry methods were devised to identify and characterise immune cell populations.

Twenty-six KTRs were randomised into one of two HIIT protocols or MICT: HIIT A (n=8; 4-, 2-, and 1-min intervals; 80-90% $\dot{V}O_{2peak}$), HIIT B (n=8, 4×4 min intervals; 80-90% $\dot{V}O_{2peak}$) or MICT (n=8, ~40 min; 50-60% $\dot{V}O_{2peak}$) for 24 supervised sessions on a stationary bike (approx. 3x/week over 8 ± 2 weeks). Blood samples taken pre-training, mid training, post-training and 3 months later. Novel multi-colour flow cytometric panels were developed to characterise lymphoid and myeloid cell population from peripheral blood mononuclear cells. No changes were observed for circulating immune and inflammatory cells over the 8-week interventions.

This feasibility study does not suggest that exercise programmes using HIIT and MICT protocols elicit adverse negative effects on immunity in KTRs. Therefore, such protocols may be immunologically safe for these patients. The inability of the participants to achieve the target exercise intensities may be due to physiological abnormalities in this population which warrants further investigation.

Keywords: High intensity interval training, Kidney transplantation, Flow cytometry, Inflammation, Immune cells

INTRODUCTION

Chronic kidney disease (CKD) is a collective term that arises from heterogeneous disorders that affect the structure and function of the kidney irreversibly, over months or years (1). The most widely used indicator of overall kidney function is glomerular filtration rate (GFR), which associates to the total amount of fluid filtered through the functioning nephrons per unit of time. In clinical settings, equations are used to estimate GFR (eGFR).

Kidney transplantation is the optimal choice of kidney replacement therapy (KRT) in terms of patient survival and quality of life (QoL). Kidney transplantations are classified as a living-donor or cadaveric-donors (deceased-donor). Regardless of the type of donor, blood type testing, tissue typing, cross-matching and serology are conducted to help match a donor to a recipient. In most cases, the patient's existing kidneys are not removed due to increases in surgical morbidity rates. Therefore, donor kidneys are often placed in the iliac fossa. The artery that carries blood to the kidney and the vein that carries blood away is surgically connected to the artery and vein already existing in the pelvis of the recipient. The urethra that carries urine from the kidney is connected to the bladder.

1.1 INCIDENCE AND PREVALENCE

CKD is a global problem and the incidence and prevalence vary greatly by nation. The most recent data puts the total

Corresponding author:
Ganisha M Hutchinson, Ph.D., Crown Bioscience UK, Hillcrest House, Dodgeford Lane, Loughborough, LE12 9TE, United Kingdom, ganisha.hutchinson@crownbio.com

number of KTRs in the UK at 32,624; a prevalence of 501 per million population (pmp) (2). During 2016, a total of 3,328 kidney transplants were performed. The absolute number of living donors had declined in 2016 however, cadaveric donors have increased between 9-13%.

The first year of transplant costs the National Health Service (NHS) £17,000 and £5000 for every subsequent year per patient. During 2016, 2.4% of prevalent transplant patients experienced graft failure and returned to dialysis (2)

The incidence and prevalence of males and females has remained stable for the past ten years. The age at which patients are transplanted has more recently been between 55-59 years old. The incidence and prevalence for KTRs for men and women in the UK was 50.9 and 53.8 years old, respectively (2).

Data suggests there being an increase in renal transplants in non-white ethnic groups in the UK. South Asian group's incidence has increased from 10.5–12.7% from 2010 to 2015 suggesting improved access to transplantation (2).

1.2 CARDIOVASCULAR DISEASE AND CKD

There has been a reduction over time of deaths in transplant patients attributed to CVD (43% in 2003 compared to 22% in 2015;(2). Transplantation has been shown to reduce the risk of CV events compared to those on dialysis (3), however outcomes still remain poorer than in the general populations (4). CVD is an overall term used for disorders of cardiovascular perfusion (e.g. atherosclerosis and ischemic heart disease) and cardiac function (e.g. congestive heart failure and left ventricular hypertrophy). Several CVD risk factors are associated with this patient group, traditional risk factors include co-morbidities whereas non-traditional are suggested to influence morbidity and mortality (table 1).

The American Heart Association considers classifying those with CKD a high-risk group for CVD (5). Although mortality rates have declined for CVD in transplantation to 18% in 2017; due to greater cardiac screening, there is still high prevalence and incidence of CVD. Inflammation has become a strong predictor of CVD and mortality (6-11). KTRs have a high activated inflammatory response due to a decrease in renal clearance of inflammatory cytokines and comorbidities as a result of their CKD resulting in immune dysfunction.

Table 1. Traditional and non-traditional risk factors for cardiovascular disease in KTRsised. This image was created with BioRender.com (Toronto, Canada).

Traditional	Non-traditional
Hypertension	Homocysteine
Dyslipidaemia	Anaemia
Diabetes mellitus	Inflammation
Renal impairment	Proteinuria
Left ventricular hypertrophy	
Lifestyle factors	

1.3 INFLAMMATION

Inflammation is a biological response of the vasculature or tissue to injury, infection, ischemia, and autoimmune diseases. Inflammation occurs through polypeptide cytokines that mediate the inflammatory response through autocrine, paracrine, and endocrine mechanisms. Chemotactic cytokines attract leukocytes and mononuclear cells to sites of injury. These cytokines are classified as either pro-inflammatory or anti-inflammatory and are involved in acute or chronic responses (12). Acute responses are non-specific short term physiological responses to injury, which can result in redness, swelling and pain (13). Chronic inflammation is referred to as slow, long term inflammation lasting months to years leading to tissue damage (9), and is associated with increases in pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6 and C-reactive protein (CRP) (14-16).

Evidence has shown inflammation to be a mediator in the prevalence of specific inflammatory diseases such as diabetes and metabolic syndrome (10, 17), heart failure and CVD (11), arthritis and joint disease (sarcopenia, osteoporosis), allergies (including infection), and depression and dementia (9, 18). Inflammation in CKD could be a consequence of decreased elimination and increased generation of pro-inflammatory cytokines as a result of decreased GFR. Regardless of the initial cause of inflammation, expression of IL-1, IL-6 (7), IL-10, IL-1 receptor antagonist (IL-1ra) (12) have been reported in experimental studies.

During the acute response, IL-6, TNF-α, interferon-γ (IFN-γ) are elevated, attributed to macrophages and monocytes at inflammatory sites. An anti-inflammatory effect can be seen following IL-6. In gene knockout mice, IL-6 caused an anti-inflammatory response both locally and systemically following endotoxin exposure (19). Whilst in humans, IL-6 infusion resulted in elevations of anti-inflammatory cytokines (IL-10 and IL-1ra) without stimulation of pro-inflammatory cytokines IL-1 and TNF-α, suggesting a protective effect (20). However, when IL-6 concentrations remain chronically high, recruitment of monocytes to the area of inflammation can cause a switch from acute to chronic responses (21). Transplant rejection accompanied with elevated serum IL-6 has been reported within 2 months following transplantation in kidney patients (22), further to this, elevated IL-6 concentrations have been reported in patients with all stages of CKD and RRT (23, 24) likely contributing to CVD (25).

TNF-α is a pro-inflammatory cytokine associated with killing tumour cells and has a role with regulating pro and anti-inflammatory cytokines (26, 27). Evidence suggests up-regulation of TNF-α in CKD patients, however the link between TNF-α and CVD mortality is controversial (27). The combination of TNF-α and IL-6 have increased pathogenesis of endothelial dysfunction. A combination of these inflammatory cytokines contributes to mortality and morbidity in KTRs (28).

The role of cytokines is to keep a balance between pro–inflammation and anti-inflammation. IL-10 is one of the most important anti-inflammatory immune-regulating cytokines. IL-10 is produced late in the activation process by T helper 2 cells and can completely prevent antigen-specific T-cell proliferation. The cytokine has a direct stimulatory effect on B-cell activation, proliferation, and differentiation into antibody secreting cells and can inhibit T helper 1 cytokine synthesis. Following pro-inflammatory cytokines, IL-10 reduces the production of chemotactic factors such as IL-8 or CC chemokines that attract further leukocytes to the site of inflammation (29). In KTRs, serum IL-10 was higher in those with impaired transplants than those with stable transplants and healthy controls (30).

1.4 IMMUNE DYSFUNCTION

Despite KTRs suffering from chronic inflammation, these patients are heavily immunosuppressed. The general principle underlying successful immunosuppression in transplantation is to keep a balance between effective prevention and rejection and signs of over-immunosuppression such as infections and malignancies (31). In 2017, it was found that the leading cause of death amongst KTRs were infection (24%) followed by malignancy (22%;(2). Today, the main form of therapy relies of multidrug combinations with synergistic components each with a different mechanism of action.

Rejection of transplants can occur in three phases: hyper acute, acute, and chronic. Hyper acute rejection involves an immediate response from the recipient's immune system against the transplanted tissue and is mainly due to actions against the donor human leukocyte antigen (HLA). Acute rejection is most frequent and is likely to occur 1 to 3 months post-transplant. Foreign antigens from the donor tissue activate the host T cells; cytotoxic T cells infiltrate the organ and begin to destroy the tissue by a release of perforins. Humoral-mediated rejection also occurs as host B-cells produce antibodies against the tissue, which results in vascular damage. Chronic rejection is a slow-developing process that gradually compromises the tissue function. A key feature in this is inflammation of the donor tissue. Activated T cells release cytokines, which recruit monocytes/ macrophages to attack the tissue, by releasing cytotoxic enzymes that inflame the transplanted tissue (32).

1.4.1 T lymphocytes

T lymphocytes originate from the bone marrow that migrate from the thymus for activation, clonal expansion, migration, effector responses and termination. Phenotypically, naïve T cells are small cells that express surface marker such as CD45RA, CCR7, CD62L, CD127 and CD132. These cells migrate within secondary lymphoid organs where they interact with dendritic cells. Effector CD4+ T cells can differentiate into four subcategories: (i) those possessing pro inflammatory effector characteristics (T helper 1 cells), (ii) those possessing regulatory or anti-inflammatory activities (regulatory T cells), (iii) those with a function to promote B cell follicle development (T helper 2 cells) and (iv) those that provide long term memory (central memory T cells).

Previous data has reported lymphopenia occurring and persisting over several years following transplantation (33). Regulatory T cells (Tregs) are classed as CD4+ cells with the expression of IL-2 receptor α chain (CD25). Tregs role of regulating and supressing immune cells via various mechanisms including the production of immunosuppressive cytokines IL-10, transforming factor growth-βeta, IL-34 and IL-35, affecting antigen presenting cell functions of dendritic cells via cell-to-cell contact. Overactivation of Tregs is associated with an increased risk of chronic infections and tumour growth; on the other hand, a deficiency can lead to autoimmunity (34), inflammation and allergy (35).

Memory T cells are defined by surface marker expression of homing receptors CCR7 and L-selectin but can be identified by CD45RO+ CCR7+ and are maintained in peripheral tissues allowing for quick responses to infection. These cells proliferate and produce cytokines in response to stimulation from antigens. It is suggested that KTRs show increases in the number of memory and central memory cells compared to end stage renal disease (ESRD) patients, likely to be attributed to an immune response following transplantation (36, 37). There is little research examining the distribution and function of T cells in KTRs.

1.4.2 Monocytes

Monocytes are a heterogeneous leukocyte type with distinct phenotypic and functional characteristics. Three human monocyte subsets exist based upon CD14 (LPS co-receptor) and CD16 (Fcγ receptor III) expression: classical CD14+CD16− (85%), intermediate CD14+CD16+ (5%) and non-classical CD14-CD16+ (10%) monocytes. Intermediate and non-classical monocytes have been distinguished as pro-inflammatory monocytes due to their capability in producing inflammatory cytokines TNF-α and IL-1β (38, 39). These subsets have been elevated in CVD patients (40) and are regarded as 'pro-atherogenic' due to their inflammatory nature (41).

Previous data has noted a shift in monocyte subsets pre and post transplantation. Increases in non-classical monocytes pre-transplantation has been associated with graft rejection compared to patients who had greater classical monocytes (42, 43). Following 6-months post-transplant a further increase in non-classical monocytes has been reported producing IFN-γ (43). Lee et al (2013) reported that, as CKD advances, the proportion of intermediate monocytes expands, and notably, the expansion of this subset correlated with measures of impaired vascular health (44).

Upon tissue damage or infection, monocytes are rapidly recruited to the affected site, where they can differentiate into macrophages. These macrophages can switch toward a classically activated phenotype (M1; pro-inflammatory phenotype) in response to Th1 cytokines (e.g., IFN-γ) or toward an alternatively activated phenotype (M2; anti-inflammatory phenotype) when exposed to Th2 factors (e.g., IL-4 and IL-13; (45)). Macrophage infiltration has been associated with both acute antibody mediated rejection and acute cellular rejection (46). The study of Grimm et al., (1999) showed that infiltration level of activated macrophages discriminates between clinical and subclinical rejection in kidney allograft patients. Presence of abundant numbers of macrophages are associated with poor graft outcome in kidney and in heart transplantation. Currently, immunosuppressive drugs; i.e. agents that inhibit the anti-donor response, can induce CD163+ M2 macrophages (47).

1.4.3 Immunosuppressive medications

Immunosuppressive medications are vital for keeping a balance between graft rejection and tolerance. These drugs have three effects, the therapeutic effect (suppressing rejection), undesired consequences of immunodeficiency (infection or cancer), and nonimmune toxicity to other tissues. This balance is vulnerable to disruption, leaving KTRs at risk and susceptible to a broad range of bacterial and viral pathogens. Viral infections can cause a 'direct' and 'indirect' effect including immune suppression predisposing patients to other opportunistic infections and oncogenesis (48). There is a wide range of immunosuppressive agents; the main drugs are shown in table 2. Currently, there is no consensus on how to tackle immunosuppressive agents, other than a trial an effect method (49).

According to the Kidney Disease: Improving Global

Outcomes (KDIGO) guidelines, using a combination of Calcineurin inhibitors and Inosine Monophosphate Dehydrogenase Inhibitors is most common. This strategy can decrease the incidence of allograft rejection by 10-20% in the first six months after transplantation (50).

The most frequently given calcineurin inhibitor is tacrolimus, which engages FK506-binding-protein 12 to create a complex that inhibits calcineurin leading to the blockage of nuclear factor of activated T cells (NFAT), which is required for the transcription of genes encoding multiple cytokines including IL-2. Mycophenolic acid is an active component of mycophenolate mofetil (MMF) and blocks the purine synthesis thereby affecting the proliferation of lymphocytes (51). Corticosteroids are also given with the combination of calcineurin and MMF that inhibits the actions of multiple cytokines such as IL-2, TNF-α and IFN-γ. The initial combination of MMF with low dose tacrolimus have shown to have high eGFR (52), reduction in serum cholesterol, LDL-cholesterol and triglycerides (53) in KTRs. However, this is a double-edge sword; the toxic effect of calcineurin inhibitors long-term is a contributor to renal failure. Incidences of diabetes mellitus (54), hyperlipidaemia (55) and anaemia (56) are common in these patients and are also undertreated. Long-term use of MMF with tacrolimus has reduced the incidence of acute rejections (57). However, there has been an increase susceptibility to infections (58), CV events (59) and malignancy, which remain frequent. T cells play a crucial role in transplant tolerance and rejection. A decline in the production of naïve T cells combined with the accumulation of memory and effector T cells, leads to a decreased diversity of the T cell repertoire resulting in an impaired response when challenged (60, 61).

Table 2. Characteristics of small-molecule immunosuppressive drugs. Adapted from Halloran, 2004

Type	Drug	Description	Mechanism
	Azathioprine	Antimetabolite pro-drug, imidazolyl derivative of 6-mercaptopurine	Converted into 6-mercaptopurine in the body to block purine metabolism and DNA synthesis
Calcineurin Inhibitors	Cyclosporine	11 amino acid cyclic peptide	Binds to cyclophilin, inhibits calcineurin phosphatase and T cell activation
	Tacrolimus	Antibiotic from Streptomyces tsukubaensis	Binds to intracellular protein, FKBP-12 to inhibit T-cell activation and IL-12 transcription
Inosine Monophosphate Dehydrogenase Inhibitors	Mycophenolate mofetil	Mycophenolic acid from penicillium molds	Blocks guanosine nucleotide synthesis, inhibits proliferation of T and B cells
Polyclonal Antibodies	Basiliximab	Glycoprotein from mouse fermentation cell line	Binds with CD25, which inhibits IL-2 binding and T cell activation
	Glucocorticoids		Inhibits inflammatory gene transcription

New combinations of immunosuppressant application and protocols are emerging, which can reduce the incidence of rejection, this is often seen as the endpoint in most trials, however the risk of organ function, drug toxicity or immune mechanisms seems to be forgotten. Robust tests for rejection that is T-cell–mediated or antibody-mediated would change clinical management and clinical trials. Measurement of immune responses could guide transplantation management in the same way that measurement of disease activity guides

other fields (e.g. the measurement of lipid levels in the management of hyperlipidemia). This suggests that long-term immunosuppression with clinical surveillance remains best for achieving successful graft and health maintenance.

Immunosuppression and immune over-activation are closely linked with KTRs going from one extreme to the other. Too much immunity can be detrimental causing autoimmunity, whereas insufficient immunity can result in disease susceptibility (16).

1.5 PHYSICAL ACTIVITY

There is strong scientific evidence that regular physical activity reduces the risk of early death, heart disease, stroke, high blood pressure, type 2 diabetes mellitus, breast and colon cancer, whilst also improving anthropometrics and mental health (62, 63). The World Health Organisation (WHO) and the American College of Sport Medicine (ACSM) strongly recommend healthy individuals to partake in physical activity to aim to improve cardiorespiratory and muscular fitness, bone and functional health, and to reduce the risk of diseases, depression and cognitive decline. It is recommended that healthy individuals should partake in at least 150 minutes of moderate intensity exercise and at least 75 minutes of vigorous intensity aerobic physical activity per week. In addition to this, weight bearing activities should be completed twice a week involving all major muscles groups (64).

1.5.1 Physical activity in kidney transplant recipients

Kidney transplantation is burdened by high CV risk because of increased prevalence of traditional and disease-specific CV factors and, consequently, patients are affected by greater morbidity and mortality (5, 65, 66). In KTRs, healthy lifestyle and physical activity are recommended to improve overall morbidity and CV outcomes (67). The International Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines for patients with kidney diseases recommend patients with CKD to undertake aerobic physical activity compatible with CV health and tolerance. It is recommended that at least 30 minutes of moderate intensity physical activity should be undertaken 5 times a week (68). However, given the KTRs are an immunocompromised population, there are no specific exercise guidelines for this population group.

Poor adherence to physical activity and low physical functioning are often a result of reduced muscle mass and/or impaired physical capacity due to co-morbidities or previous effects of dialysis treatment (69). Further specific factors related to kidney transplantation itself, such as renal failure, immunosuppressive treatment leading to myopathy and myalgia resulting in muscle weakness (65), obesity, diabetes, may contribute to the impairment of physical performances in transplanted patients.

1.5.2 Exercise training in CKD and kidney transplant recipients

Transplanted populations are often disregarded from studies and excluded from most physical therapies, hence there is a lack of evidence regarding exercise training and specific guidelines for this population group (70, 71).

1.5.2.1 Exercise type, intensity, and duration

It is well known that physiological adaptations of the CV system and muscle from exercise are dependent on exercise type, intensity and duration (26, 72). Reviews have described these finding in further depth (73-75). The most common form of exercise training used in CKD and heart transplantation research is CV exercise which has increased aerobic capacity and heart rate variability in CKD stages 3-5, haemodialysis and KTRs (76-79). Other exercise training programmes have used a combination of CV and resistance exercise (80-82), as well as solely resistance training (83-85).

Percentage V̇O2peak, heart rate peak (HRpeak) or the rating of perceived exertion (RPE)-Borg scale have been used to define exercise intensity for interventions. The majority of studies use a moderate to high intensity protocol between 60-80% HRpeak to induce physiological and muscle adaptations (76, 83, 84, 86, 87). Evidence of this intensity has shown to improve triglyceride/ high density lipoprotein concentrations (88). Regular exercise can be advantageous to reduce symptoms of renal skeletal muscle disorders (89-91). KTRs have an impaired exercise capacity possibly related to immunosuppressive medications inducing muscle atrophy (92, 93). Exercise time on treadmills test, isokinetic muscle power and muscle endurance are reported to increase after 3 months of training, suggesting that skeletal muscle contractile function increases with training in KTRs (92). It is also reported that training counteracts some of the negative effects of glucocorticoid therapy, such as muscle wasting and bodyweight gain, bone metabolism, favouring bone remodelling and may reduce the need for medication to control hypertension (92, 94).

It appears to be clear that exercise regardless of type, intensity or duration has potential benefits to all CKD patients improving health and wellbeing. However, the specific mechanisms behind improvement are not well reported.

Majority of studies report moderate intensity exercise for improvements in CV risk factors and exercise capacity in CKD populations (74, 95-97). Aerobic and resistance based exercise have been shown to be feasible in this population for physical and psychological rehabilitation (98). An evidence based review in solid organ transplant patients assesses moderate to vigorous intensity exercise in patients for improvement in exercise capacity and muscle strength (99), however, there is little to no data for the effects of HIIT on immune and inflammatory markers.

1.5.3 High intensity interval training

High intensity interval training (HIIT) is physical exercise that is characterised by brief, intermittent bursts of vigorous activity, interspersed by periods of rest or low-intensity exercise. Previous models of HIIT used four to six Wingate tests, which involves 30 seconds all-out maximal cycling – separated by four minutes of recovery, totalling only 2 to 3 minutes of very intense exercise per training session (100-103). Reports of improvements in exercise capacity and peripheral adaptations have been reported (104, 105). HIIT has been documented to allow for greater physiological stimulus and adaptations than MICT. These larger benefits seen in the healthy populations are for cardiorespiratory fitness (100, 101, 103), vascular function (106), and skeletal muscle metabolism (101).

More recently, HIIT protocols have been widely used in clinical populations, although protocols may differ to those previously reported. The most widely used HIIT protocol in clinical populations is the 4 x 4 minute at 80-90% HRpeak interspersed with 3-minute periods consisting at 50-60% HRpeak (106-108). This protocol has been deemed safe and tolerable in heart failure and metabolic diseased populations

with improvements in exercise capacity, endothelial function, glucose control and fat metabolism compared to MICT (109, 110). There have been few reports of using HIIT and MICT in CKD populations (111) and haemodialysis patients (97), with improvements in exercise capacity and skeletal muscle synthesis (111). There is superiority in HIIT protocols over MICT in promoting health benefits in diseased populations (107, 112). It has been reported that patients with cardiometabolic disease found an increase in V̇O2peak after HIIT compared to MICT (107), which can be translated as a greater decrease in risk of morbidity and all-cause mortality. Reductions in blood pressure, improved insulin sensitivity, increased nitric oxide availability, improvement in lipid metabolism and increases in PGC-1α have also been reported (107). However, with these studies the sample sizes are often too small, and the length of training is often short. Randomised control trials (RCTs) that compare the superiority of multiple different HIIT protocols in improving aerobic and metabolic parameters is limited (113, 114). Thus, researchers tend to pick a protocol that they can modify the work rest times. More studies are warranted to establish the most efficient protocol for each target subject according to clinical characteristics and fitness level, to improve aerobic capacity and to establish higher adherence. Although the increased application of HIIT in the health and medical fields is expected, its feasibility and safety should be further evaluated in future.

1.5.4 Exercise and inflammation

In the general population, evidence suggests that markers of systemic inflammation are lower in physically active individuals than sedentary individuals (115). Studies have observed an inverse relationship between inflammatory markers and physical activity levels (116).

1.5.4.1 Acute inflammation

A single bout of exercise initiates an inflammatory process in the circulation similar to those induced by infection, sepsis, or trauma (117). There is an increase in the number of circulating leukocytes (mainly neutrophils, lymphocytes, and monocytes) in relation to the intensity and duration of the exercise bout. Increases in inflammatory plasma cytokine concentrations are seen that influence leukocytes such as TNF-α, MCP-1, and IL-1β; and anti-inflammatory cytokines IL-6, IL-10 and IL-1ra. Muscle derived IL-6 increases with exercise and contributes to an increase in circulating levels of IL-6 (118) . Reports in healthy populations have found increases in plasma IL-6 after 30 minutes of running (119) with increases in concentrations up to 100-fold after a marathon (120). It was recently demonstrated that relatively small increases in plasma levels of IL-6 induce the two anti-inflammatory cytokines IL-1ra and IL-10, together with CRP (20) . In CKD populations, moderate intensity exercise has shown to increase concentrations of IL-6 post exercise that have remained elevated up to 1-hour post exercise bout with increases in TNF-α (96, 121). Further data in middle aged participants have found increases in IL-6, IL-8, IL-10 and TNF-α following a single bout of moderate intensity exercise (122).

Humoral or cell-mediated immunity is dependent on the type of cytokines released by T helper cells. Type 1 cells produce IFN-γ and TNF-α which activate macrophages and induce killer mechanisms which include cytotoxic T cells which protect against intracellular pathogens. Whereas type 2 cells produce IL-4, IL-5, IL-10, and IL-13 which promote humoral immunity. Strenuous exercise decreases the percentage of type 1 cells within the circulation, whereas type 2 cells do not change (123). Acute exercise has been shown to decrease the proliferative lymphocyte responses to mitogens (124). With the number of lymphocytes decreasing below pre exercise levels and the CD4+/CD8+ ratio decreasing (125). However, this was not seen in CKD patients after a single bout of walking (121). The T cell repertoire changes following an acute bout of exercise. Both memory and naive T cells increase during exercise, but the mobilisation of memory T cells is greater (125). Many studies have examined the influence of acute exercise on monocytes populations and have typically reported monocytosis following exercise with shifts in monocyte phenotypes to CD14+/CD16+ subsets within the circulation that exhibit pro-inflammatory properties (126, 127) in healthy populations and CKD and clinical populations (128).

1.5.4.2 Chronic inflammation

It is now evident that an acute inflammatory response plays a major role in training adaptations. Regular exercise can lead to lower basal levels of circulating inflammatory markers, as well as reduce the inflammatory response to acute exercise. In CKD populations, 6-months of regular walking resulted in a reduction of basal IL-6, and IL-10 elevations compared to non-exercising controls. This also led to downregulation of lymphocyte and monocyte activation and improvements in systemic inflammation (121).

Adipose tissue can play a role in chronic inflammation through two to three fold increases in plasma concentrations of "adipokines" (TNF-α, IL-6, monocyte chemoattractant protein (MCP) -1, CRP) (12). When under metabolic stress, the secretion of adipocytes results in the recruitment and activation of immune cells. Monocytes and T cells primed by inflammatory molecules TNF-α and IFN-γ lead to monocyte-T cell cross activation (14). The inflammation caused by immune cell cross talk is not a consequence of immune cell infiltration but a consequence of immune cell adipocyte interaction (14). Exercise can have a beneficial effect on adiposity. Non-obese individuals that are inactive have elevated plasma markers of inflammation compared to non-obese active individuals (10, 17, 129), and it appears that the mechanisms through which increases in physical activity has a positive impact upon inflammation are not restricted to changes in adiposity. Observational and interventional studies in the general population and clinical populations have noted lower inflammatory biomarker concentrations in individuals who report performing more frequent and more intense physical activity including lower IL-6 and CRP levels (130-133) . However, interventional study data is less consistent, likely because of lack of control groups, small sample sizes, differences in exercise intensities. Potential mechanisms by which exercise may reduce inflammation may include shifts in monocyte phenotype, especially reductions in immune cell production of inflammatory mediators and immune function adaptations that occur locally in exercised skeletal muscle. Studies have noted in clinical populations, exercise training to reduce intermediate monocyte number as well as TNF-α production (134)

This manuscript is generated from two data sets; The PACE-KD study and the MAP-KD study. The PACE-KD

study's primary aim was to assess the recruitment, retention and adherence to HIIT and MICT programmes in KTRs (manuscript under review). Here we report a secondary analysis aiming to assess the effects of 8-weeks of HIIT and MICT on circulating measures of immunity and inflammation in KTRs. The MAP-KD study was to compare both immune and inflammatory cells in stable KTRs and non-CKD controls over a longitudinal period of 6-months using flow cytometry to assess immune cells and sandwich-based assays to assess cytokine concentrations. A comparison of the two groups may generate comparative values of cell subsets that may be used as a reference tool for graft tolerance and rejection in KTRs.

METHODS

The data included in this report were derived from two clinical studies: PACE-KD (135), a feasibility study of HIIT and MICT training in KTRs, and MAP-KD, a longitudinal observational study assessing circulating immune and inflammatory parameters in KTRs and non-CKD as control data for changes over time. Briefly, in the PACE-KD study KTRs were randomised to 24 thrice weekly supervised HIIT or MICT sessions, matched for workload. Venous blood samples were taken at defined timepoints. In MAP-KD there was no intervention, but venous blood samples were obtained at the same timepoints for comparison. Approval for both studies was granted from the UK National Research Ethics Committee (PACE-KD: 18/EM/0031; MAP-KD: 16/EM/0482). All participants gave written informed consent, and the trial was conducted in accordance with the Declaration of Helsinki. The studies were registered with ISRCTN (PACE-KD: no. 73106458, MAP-KD no. 17122775).

2.1 PARTICIPANTS

All KTRs were recruited from routine kidney transplant outpatient clinics at Leicester General Hospital, United Kingdom. Patients were excluded if they were scheduled surgery or procedures involving anaesthesia, pregnancy, significant disease, or disorder which may put the patient at risk while taking part in the study or may influence the results of the study, and the inability to give informed consent or comply with the testing and training protocol. Eight KTRs were recruited for the MAP-KD study and eight non-CKD individuals known to the research team (i.e., research staff, university and hospital staff or patient family members) were recruited as non-kidney disease controls. Outcome measures were taken at pre-training, mid-training, post-training and 3-months post training for the PACE-KD study, and the same time points for the MAP-KD study: Week 0, Week 4, Week 8 and Week 24.

2.2 EXERCISE TRAINING INTERVENTION

The PACE-KD study exercise intervention has been described in detail previously (135). Briefly, 24 KTRs were randomised to one of two HIIT protocols or MICT: HIIT A (n=8; 4-, 2-, and 1-min intervals; 80-90% $\dot{V}O_{2peak}$), HIIT B (n=8, 4×4 min intervals; 80-90% $\dot{V}O_{2peak}$) or MICT (n=8, ~40 min; 50-60% $\dot{V}O_{2peak}$) for 24 supervised sessions on a stationary bike (approx. 3x/week over 8 ± 2 weeks) and 3 months after the last session.

2.3 BLOOD SAMPLING, HANDLING, AND ANALYSIS

Blood samples were taken on four occasions (pre-training, mid-training, post-training and 3-months post-training).

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Ficoll-Plaque (GE Healthcare, Merck, St Louis, MO USA) (136). The cells were washed twice with sterile PBS. Cells were resuspended in 1mL of complete Dulbecco's Modified Eagle Medium (D-MEM) with 10% heat inactivated fetal bovine serum [500mL D-MEM, 10% fetal bovine serum, 5mL L-glutamine (100mM), 5mL HEPES buffer (1M), 5mL sodium pyruvate (100mM), 10mL MEM amino acids (50X) and 5mL penicillin/ streptomycin (10,000U/ 10mg/mL); Merck, St Louis, MO USA]. One millilitre of freezing media composed of 20% dimethyl sulfoxide (DMSO, Merck, St Louis, MO USA) [40mL heat inactivated fetal bovine serum, 20% DMSO] was then added slowly to the cell suspension. The cells in freezing media were aliquot at 2 x 10^6 cells per mL in 2mL Corning freezing vials (ThermoFisher Scientific, Waltham, MA USA). A CoolCell freezing device (Biocison, Merck, St Louis, MO USA) was used to freeze cell aliquots at -80°C for 24 hours where after cells were stored at -150°C awaiting future analysis (136).

2.3.1 Thawing peripheral blood mononuclear cells

Cell aliquots were thawed at 37°C until a small ice slurry remained. Complete D-MEM was pre-warmed to 37°C, 1mL of pre-warmed media was added to the cell aliquot slowly and the whole was transferred into 15mL conical tube. Cells were washed with 10mL pre-warmed media twice and centrifuged at 400 x g for 10 minutes. The supernatant was decanted, and cells re-suspended in PBS.

2.3.2 Staining peripheral blood mononuclear cells

Firstly, cells were stained with live/dead fixable viability stain (Thermo Fisher Scientific, Waltham, MA USA) by adding the dye to cells suspended in PBS. Cells were incubated for 30 minutes at 4°C protected from light. Cells were then washed in PBS and resuspended in BD staining buffer (BD Bioscience, San Jose, CA USA) and aliquoted into a 96-well U bottomed plate (Greiner CellStar, Merck, St Louis, MO USA). Briefly, BD stain buffer and 2.5µL human Fc blocker (BD Bioscience, San Jose, CA USA) was added to each well and incubated for 10 minutes at room temperature, protected from light. All wells were stained with the relevant antibodies and incubated at 4°C for 30 minutes, protected from light throughout. Cells were washed with flow staining media [500mL Hanks' Balanced Salt Solution, 25mL fetal bovine serum, 5mL L-glutamine (100mM), 5mL HEPES buffer (1M), 5mL sodium pyruvate (100mM), 10mL MEM amino acids (50X) and 5mL penicillin/ streptomycin (10,000U/ 10mg/mL), Merck, St Louis, MO USA] and centrifuged at 200 x g for 5 minutes. Supernatants were discarded; and cells resuspended in media and fixed with formaldehyde solution (Merck, St Louis, MO USA). Cells were transferred into microtubes for flow cytometry analysis.

2.3.3 Flow cytometry collection and analysis

A BD FACSCelesta cytometer with three lasers was used for the study. The instrument configurations were optimised for 12 fluorescence parameters. The blue laser (488nm) stimulated four fluorochromes: PerCP-Cy 5.5 detected via 695/40 bandpass; PE-CF594 (610/20 bandpass); PE (575/25 bandpass) and FITC (530/30 bandpass). The red laser (640nm) stimulated three fluorochromes: APC-CY7 (760/60 bandpass);

APC (670/30 bandpass) and Alexa Fluor 700 (730/45 bandpass). The violet laser (405nm) stimulated five fluorochromes: BV786 (780/60 bandpass); BV650 (670/30 bandpass); BV605 (610/20 bandpass); BV510 (525/50 bandpass) and BV421 (450/40 bandpass). Alternate fluorochromes could be used given the same excitation and band pass filters.

For the 10-colour lymphocyte flow cytometry panel, the following antibodies were used: CD127-PerCP-Cy5.5 (clone SB/199, Biolegend, San Diego, CA USA); CD56-PE (clone CMSSB, Thermo Fisher Scientific, Waltham, MA USA); CD8a-FITC (clone RPA-T8, BD Bioscience, San Jose, CA USA); CD4-APC-Cy7 (clone RPA-T4, Cambridge Bioscience, Cambridge UK); CD3-AF-700 (clone UCHT1, Thermo Fisher Scientific, Waltham, MA USA); CD45RA-APC (clone HI100, Thermo Fisher Scientific, Waltham, MA USA); CD19-BV786 (clone HIB19, BD Bioscience, San Jose, CA USA); CD25-BV650 (clone BC96, Biolegend, San Diego, CA USA); CD45RO-BV605 (clone UCHL1, BD Bioscience, San Jose, CA USA); fixable aqua viability stain- BV510 (Thermo Fisher Scientific, Waltham, MA USA).

For the 4-colour monocyte flow cytometry panel the following antibodies were used: CD14-PerCP-Cy5.5 (clone M5E2, BD Bioscience, San Jose, CA USA); HLA-DR-APC-H7 (clone G46-6, BD Bioscience, San Jose, CA USA); CD16-BV650 (clone 3G8, BD Bioscience, San Jose, CA USA) and fixable aqua viability stain- BV510 (Thermo Fisher Scientific, Waltham, MA USA).

The flow cytometer was calibrated each day prior to sample acquisition using the cytometer setup & tracking kit (CS&T) and rainbow fluorescent 8-peak bead particles (both BD Bioscience, San Jose, CA USA). Data was acquired using instrument installed BD FACSDiva (BD Bioscience, San Jose, CA USA) software and samples were gated based on FSC and SSC parameters followed by live/dead discrimination. The flow cytometry standard (FCS) files were further analysed using FlowJo v10.1 software (FlowJo, LLC). Stained PBMCs which fell within the size FSC and granularity SSC appropriate for leukocytes, were further gated for doublet population and finally cells were gated based on FSC-height and viability before entering full analysis. These steps reduced spurious results and provided confidence in the subsequent analysis.

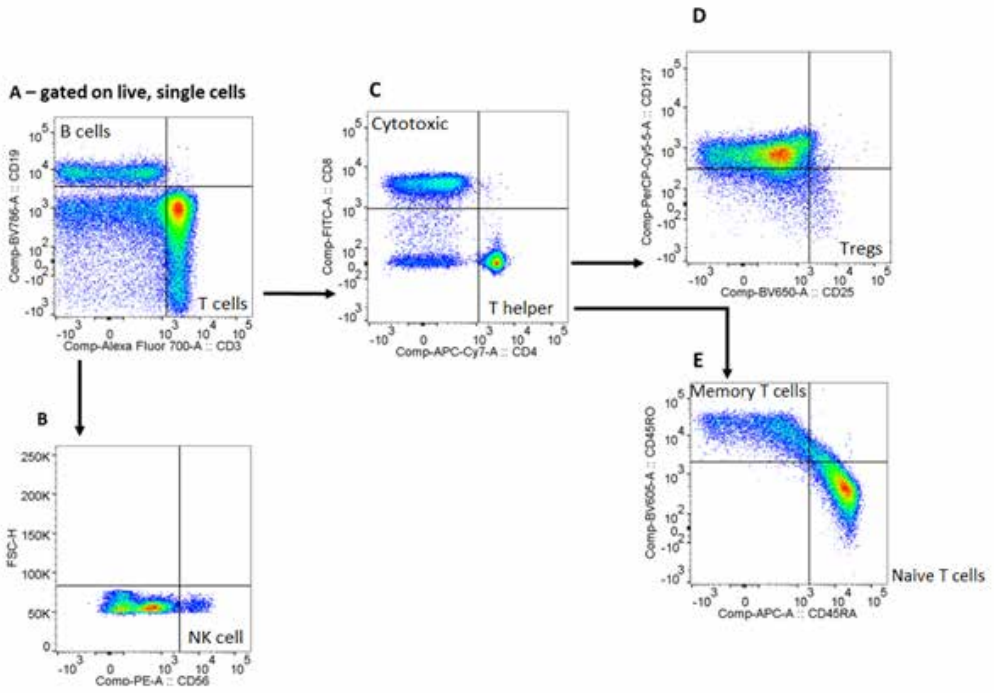

Figure 1. Demonstration of gating strategy used to identify T cell subsets using FlowJo v10.1. Cells are identified as lymphocytes based on size and granularity; single cells are selected based on doublet discrimination. Live, single cells are gated using a fixable viability dye, T cells (CD3+) in the lower right quadrant, B cells (CD3-CD19+) in the upper left quadrant (A), NK cells (CD3-CD56+) in the lower right quadrant (B), gating helper CD4+ T cells in the lower right quadrant and cytotoxic CD8+ in the upper left quadrant (C), initial regulatory T cells (CD25+CD127- (D), naïve (CD45RA) and memory (CD45RO) T cells are gated from CD4+ cell (E).

2.3.4 Lymphocyte analysis

To initially characterise discrete populations within the PBMCs, FSC and SSC properties followed by FSC height and area were used to identify single lymphocytes, from this population BV510 Aqua was used to detect dead cells, all live single cells were gated for further analysis. The single, size and granularity defined, live cells were gated based on CD3 and CD19 expression thereby providing broadly defined B cell (CD19) and T cell (CD3) populations. The single live lymphocyte population that was negative for both CD3 and CD19 was further assessed for expression of CD56 to define a natural killer (NK) population. The single, live lymphocytes expressing CD3 were further analysed for CD4 and CD8 expression to indicate MHC class II and MHC class I restricted T cells, respectively. To address function of the cells CD4+ expressing cells were examined for levels of the receptors for IL-7 (CD127) and IL-2 (CD25) receptors to identify the CD25+/CD127- cells which are thought to represent a regulatory T cell population in humans (Liu et al., 2006). The live single CD3, CD4 expressing T cells were also assessed to determine whether they had been exposed to antigen with CD45RA expression representing naïve T cells and CD45RO representing antigen experienced cells (figure 1; (137).

2.3.5 Monocyte analysis

Cellular differentiation of myeloid populations was achieved using FSC and SSC properties. The viability dye and FSC height and area were used to exclude dead cells and any doublet populations as discussed above. To reduce the complexity of the populations under examination first BV510 conjugated CD3, CD19 and CD56 antibodies were used to place T cells, B cells and NK cells within the same gate as cells staining as non-viable. The remaining cells that expressed both HLA-DR and CD14 were myeloid cells for further analysis. These cells were assessed for their expression of the LPS receptor (CD14) and FcγRIII (CD16) which enable activation, degranulation, phagocytosis, and oxidative burst (138). These populations

are split into three monocyte subsets, non-classical monocytes (CD14-CD16+), intermediate monocytes (CD14+CD16+), the smallest of monocyte sub-population and classical monocytes (CD14+CD16-) which dominate the peripheral circulation (139) (figure 2).

2.3.6 Plasma extraction and analysis

EDTA-treated whole blood was centrifuged at 2500 x g for 10 minutes at 4°C. The plasma obtained was aliquoted into Eppendorf tubes (ThermoFisher Scientific, Waltham, MA USA) and stored at -80°C for later analysis. Plasma samples were analysed by Affinity Biomarker Laboratory (London, United Kingdom) using custom made V plex proinflammatory panel measuring IL-6, IL-10 and TNF-α (Mesoscale Discovery, MD USA), according to the manufacturer's instructions.

3 RESULTS

3.1 BASELINE CHARACTERISTICS

Individual characteristics of KTR and non-CKD controls that are included in the comparative analysis (MAP-KD study) can be found in table 3. Patient characteristics of KTRs who took part in the exercise intervention study PACE-KD can be found in table 4.

3.2 THE MAP-KD STUDY

3.2.1 eGFR and leukocyte counts

eGFR was significantly lower in KTRs than non-CKD controls at all timepoints; there was no change over 6-months for eGFR in KTRs or non-CKD controls. KTRs had higher white blood cell (WBC) counts than non-CKD controls at 6-months. Neutrophil counts were greater in KTRs than non-CKD controls at week 4 and 6-months. No differences were found between or within groups for lymphocyte or monocyte counts (P>.05; table 5).

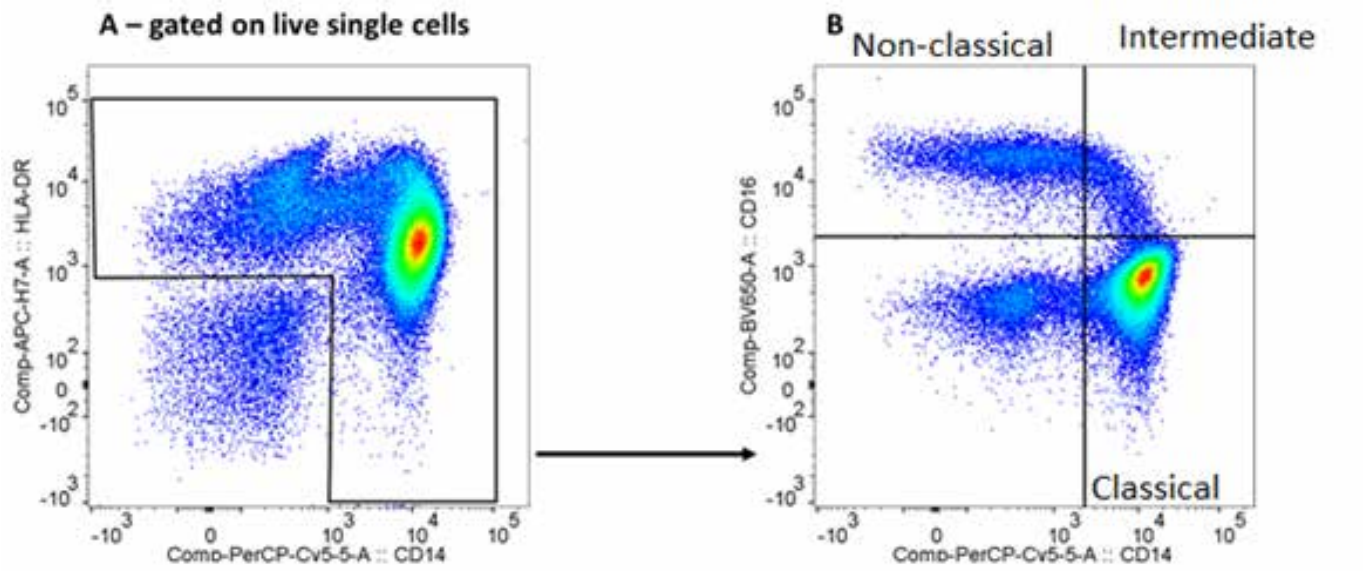

Figure 2. Demonstration of gating strategy used to identify myeloid cells using FlowJo V10.1. Cells are identified to be monocytes based on size and granularity; single cells gated for doublet discrimination, live cells are gated for analysis, CD3+, CD19+ and CD56+ cells are excluded from the analysis. HLA-DR+CD14- cells are gated to remove any other population (A). Monocyte subsets: upper left quadrant non-classical (CD14-CD16+), upper right quadrant intermediate (CD14+CD16+), lower right quadrant classical (CD14+CD16-; B).

2.3.7 Statistical analysis

Data are presented as median (Mdn) and interquartile range (IQR) unless stated otherwise. Participant characteristics were examined using a one-way analysis of variance (ANOVA) to assess differences between the groups at pre-training. Data was inspected for normality using the Shapiro-Wilk test. For non-normally distributed data a non-parametric equivalent was used as appropriate. Effect sizes (ES) are provided on all significant data to give an indication of the proportion of the variance attributed to groups using Rosenthal's definition r = 0.10, 0.30, 0.50 representing 'small', 'medium' and 'large' effects, respectively (140). All statistical analysis was performed on Statistical Package for Social Sciences (SPSS v.25, IBM, NY, USA). Graphs were drawn using GraphPad Prism (v7. GraphPad Software Inc, CA, USA). Statistical significance was accepted at the P < .05 level.

3.2.2 Markers of cellular inflammation

The absolute number of cells and the proportion of cells are given for each marker.

Lymphocyte subsets (figure 3)

T lymphocytes

Non-CKD controls had higher absolute CD3+ cell counts than KTRs at 6-months (P=.040, ES=-0.53). No change for CD3+ cells were seen for KTRs and non-CKD controls over 6-months.

There were no differences observed between KTRs and non-CKD controls for the proportion of CD3+ lymphocytes at week 0, week 4, week 8 and 6-months. However, the proportion of CD3+ cells decreased at 6-months compared to week 0 for non-CKD controls (P=.012, ES=-0.95).

Table 3. Participant characteristics for MAP-KD (n = 16)

	KTRs (n=8)	Non-CKD (n=8)	*P* value
Sex (males)	5 (63)	2 (25)	.149
Age (years)	54 ± 12	42 ± 19	.139
Weight (kg)	78 ± 24	70 ± 11	.407
Height (cm)	173 ± 15	170 ± 11	.609
BMI ($kg \cdot m^2$)	26 ± 5	24 ± 4	.576
eGFR ($ml \cdot min \cdot 1.73m^2$)	60 ± 12	>90	<.0001*
Time since transplant (months)	84 ± 60	n/a	n/a
Medications			
CNI	8 (100)	n/a	n/a
Steroid	2 (25)	n/a	n/a
Antihypertensive	7 (88)	n/a	n/a
Diabetes	3 (38)	n/a	n/a
Statins	8 (100)	n/a	n/a

Abbreviations: BMI; body mass index, eGFR; estimated glomerular filtration rate, CNI; calcineurin inhibitor
Data are presented as mean ± SD
Notes: categorical values are expressed as integer (% of n)
*denotes significant difference KTRs eGFR < non-CKD controls eGFR (P < .05)

B lymphocytes
No differences were observed between or within groups for the absolute number or proportion of CD19+ lymphocytes.

NK cells
The absolute number and proportion of CD56+ cells for KTRs decreased week 4 (P=.016, ES=-0.84) and at 6-months (P=.016, ES=-0.89). There were decreases for absolute CD56+ cells for non-CKD controls at week 8 (P=.008, ES=-0.89) and 6-months (P=.008, ES=-0.89), but not for the proportion of cells.

T helper cells, Cytotoxic T cells and Regulatory T cells (figure 4)
Non-CKD controls had greater number of CD4+ helper cells then KTRs at 6-months (P=.006, ES=-0.68). There were no significant differences over 6-months for either group. The proportion of CD4+ helper cells were greater in non-CKD controls than KTRs at week 4 (P=.040, ES=-0.29) and 6-months (P=.040, ES=-0.53). There were no changes over 6-months for KTRs and non-CKD controls.

No differences for the absolute number of CD8+ cytotoxic cells were observed between or within groups. The proportion of CD8+ cytotoxic cells were greater in KTRs than non-CKD controls at 6-months (P=.021, ES=-0.59). CD8+ cytotoxic cell frequency decreased at 6-months for non-CKD controls (P=.012, ES=-0.95).

There were no differences for the absolute number or proportion of regulatory T cells between the groups at week 0, week 4, week 8 or 6-months. Regulatory T cells decreased at 6-months for KTRs (P=.016, ES=-0.89) and decreased for non-CKD controls at week 8 (P=.008, ES= 0.89) and 6-months (P=.008, ES=-0.89). The proportion of regulatory T cells decreased at week 8 for KTRs (P=.0.12, ES=-0.89) and non-CKD controls (P=.0.12, ES=-0.89) and at 6-months for non-CKD controls (P=.012, ES=-0.89).

Naïve and Memory T cells (figure 5)
No differences were observed for absolute number or proportion of naïve T cells between or within groups. No differences were observed between groups for the absolute number or proportion of memory T cells. The absolute and proportion of CD45RO+ cells decreased at 6-months for KTRs (P=.016, ES=-0.89).

Monocyte subsets (figure 6)
There were no differences in the absolute number or proportion of non-classical and classical monocyte subsets within or between groups. The absolute number of intermediate monocytes decreased for KTRs at 4 weeks (P=.008, ES=-0.89), but no changes were observed for non-CKD controls.

3.2.3 Markers of circulating inflammation (table 6)
IL-6 concentrations were greater in KTRs than in non-CKD controls at week 0 and week 4. There was no difference for

Table 4. Participant characteristics for PACE-KD

Variable	All (N=24)	HIIT A (n=8)	HIIT B (n=8)	MICT (n=8)
Sex (male)	16 (67)	3 (38)	6 (75)	7 (88)
Ethnicity				
White	18 (75)	5 (63)	5 (63)	8 (100)
Indian	3 (13)	2 (25)	1 (13)	0 (0)
White-Black Caribbean	1 (4)	0 (0)	1 (13)	0 (0)
African	1 (4)	0 (0)	1 (13)	0 (0)
Pakistani	1 (4)	1 (13)	0 (0)	0 (0)
Body mass (kg)	79.7 ± 19.7	68.5 ± 15.6	84.1 ± 24	86.5 ± 15.5
BMI	27.2 ± 5.6	25.9 ± 5.4	28.5 ± 7.1	27.1 ± 4.3
$\dot{V}O_{2\ peak}$ (L/min)	1.99 ± 0.70	1.56 ± 0.30	2.09 ± 0.86	2.33 ± 0.65
$\dot{V}O_{2\ peak}$ (mL/kg^{-1}/min^{-1})	25.28 ± 7.48	23.69 ± 6.29	24.65 ± 7.67	27.5 ± 8.73
Systolic blood pressure (mmHg)	133 ± 14	127 ± 11	135 ± 17	137 ± 13
Diastolic blood pressure (mmHg)	85 ± 9	86 ± 6	81 ± 6	88 ± 11
eGFR (mL/min/1.73 m^2)	58 ± 19	62 ± 18	57 ± 22	55 ± 19
Serum creatinine (mmol/L)	125 ± 49	104 ± 27	133 ± 60	138 ± 53
Kidney transplant vintage (months)†	35 ± 52	12 ± 20	44 ± 86	44 ± 35
Medication				
CNI	24 (100)	8 (100)	8 (100)	8 (100)
Steroid	11 (46)	6 (75)	2 (25)	3 (38)
Anti-hypertensive	22 (92)	7 (88)	7 (88)	8 (100)
Anti-Diabetes	5 (21)	1 (13)	3 (38)	1 (13)
Statins	15 (63)	4 (50)	5 (63)	6 (75)

Abbreviations: CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; HIIT, high intensity interval training; MICT, moderate intensity continuous training
Notes: Unless otherwise indicated, values for categorical variables are expressed as integer (% of n); values for continuous variables as mean ± SD. †median (IQ), data is non-significant between groups

IL-6 concentrations in KTRs or non-CKD control over 6 months. There were no differences between or within groups for IL-10 concentrations. TNF-α concentrations were greater in KTRs than in non-CKD controls at week 0, week 4, week 8 and at 6-months. There was no difference for TNF-α concentration over time for KTRs or non-CKD controls.

3.3 THE PACE-KD STUDY

3.3.1 Leukocyte counts

Total leukocyte counts did not differ between groups pre-training or post training. In the HIIT A group, total WBC significantly increased from pre-training to post-training. Conversely, total leukocyte counts in HIIT B and MICT did not change from pre to post-training. There were no changes between groups for neutrophil, lymphocyte, or monocyte counts (P>.05).

Table 5. Summary of eGFR data (mL.min.1.73m^2) and leukocyte data (x10^9/L) at Week 0, Week 4, Week 8 and 6-months for KTRs and healthy controls

Table 5. Summary of eGFR data (mL.min.1.73m^2) and leukocyte data (x10^9/L) at Week 0, Week 4, Week 8 and 6-months for KTRs and healthy controls

	KTRs	Non-CKD		
	Mdn [IQR]	Mdn [IQR]	ES	P value
eGFR				
Week 0	59 [49 - 72]	> 90	-0.86	< .001*
Week 4	65 [52 - 70]	> 90	-0.80	< .001*
Week 8	67 [55 - 86]	> 90	-0.80	< .001*
6-months	61 [52 - 70]	>90	-0.69	< .001*
Total WBC				
Week 0	6.3 [5 - 8]	5.1 [4 - 6]	-0.34	.172
Week 4	7.9 [6 - 9]	5.0 [4 - 7]	-0.36	.084
Week 8	6.2 [5 - 8]	5.2 [4 - 6]	-0.39	.112
6-months	7.4 [6 - 8]	5.0 [4 - 6]	-0.64	.025*
Neutrophils				
Week 0	3.7 [2.7 - 4.5]	2.5 [1.9 - 3.5]	-0.36	.154
Week 4	4.8 [3.3 - 5.1]	2.8 [2.1 - 4.2]	-0.56	.038*
Week 8	3.8 [2.2 - 5.1]	2.7 [2.2 - 3.5]	-0.26	.315
6-months	4.4 [3.1 - 5.4]	2.5 [2.2 - 3.5]	-0.69	.015*
Lymphocytes				
Week 0	2.1 [1.5 - 2.5]	1.8 [1.6 - 2.1]	-0.18	.505
Week 4	1.9 [1.5 - 2.3]	1.5 [1.6 - 2.0]	-0.15	.620
Week 8	2.2 [1.3 - 2.3]	1.8 [1.4 - 2.0]	-0.23	.367
6-months	1.7 [1.3 - 2.0]	1.6 [1.4 - 1.8]	-0.18	.589
Monocytes				
Week 0	0.4 [0.3 - 0.5]	0.3 [0.1 - 0.4]	-0.29	.262
Week 4	0.4 [0.4 - 0.5]	0.3 [0.3 - 0.4]	-0.51	.059
Week 8	0.3 [0.3 - 0.5]	0.3 [0.3 - 0.4]	-0.26	.314
6-months	0.4 [0.3 - 0.6]	0.3 [0.3 - 0.4]	-0.41	.177

KTR; Kidney transplant recipients (n = 8), non-CKD control (n = 8), non-normally distributed data presented as median and interquartile range; Mann-Whitney U test between KTRs and healthy controls, ES; effect sizes, P < .05
*denotes significant difference between groups

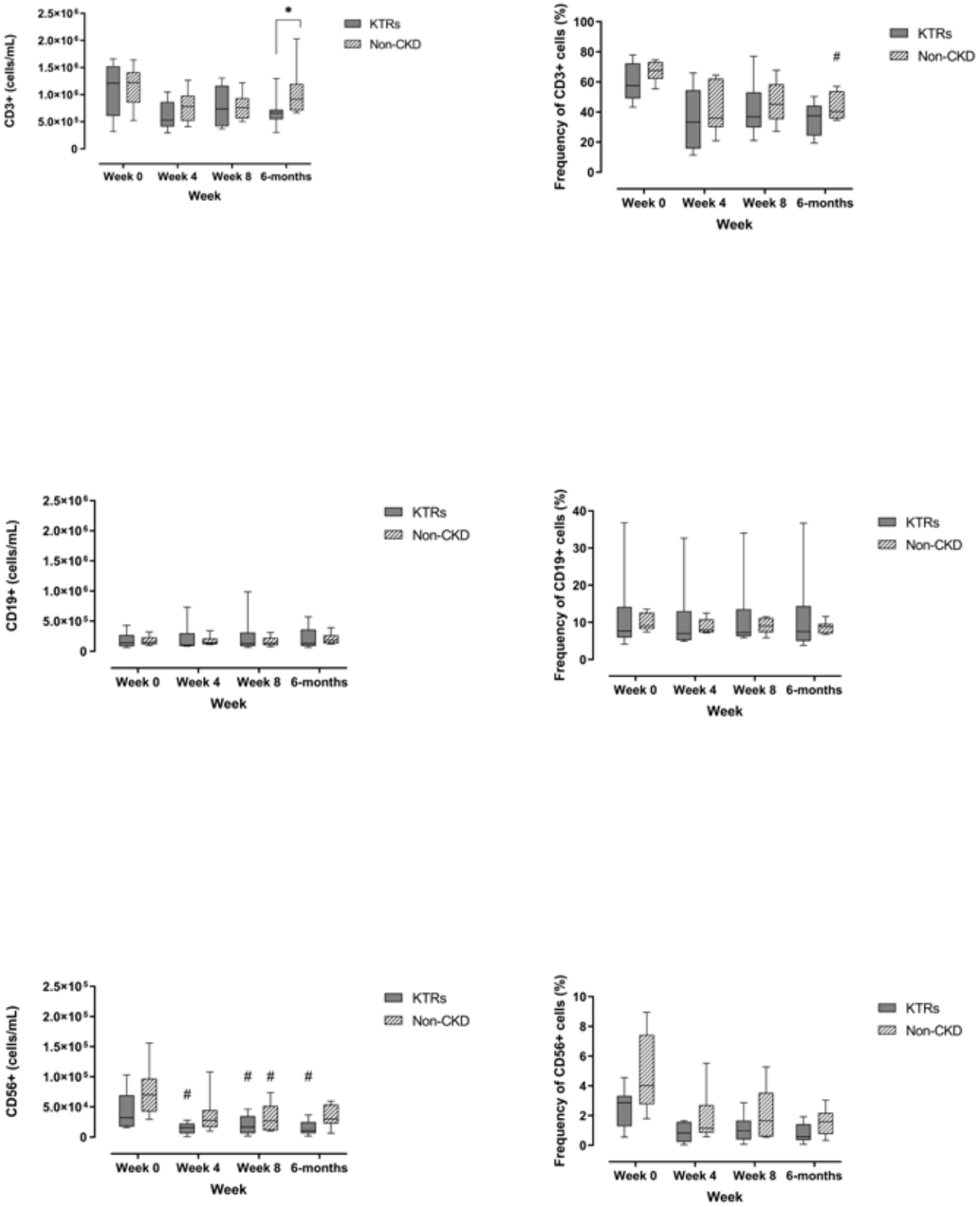

Figure 3. Cell numbers (cells/mL) and cell proportion (%) for CD3+, CD19+, CD56+ cells at week 0, week 4, week 8 and 6-months for KTRs and Non-CKD controls KTR; kidney transplant recipients, non-CKD; non-chronic kidney disease, non-normally distributed data presented as median and interquartile range. # Denotes significant difference from week 0 for Non-CKD
* Denotes a significant difference between groups, non-CKD greater KTRs

Table 6. Summary of cytokine data (ng/L) at Week 0, Week 4, Week 8 and 6-months for KTRs and non-CKD controls

	KTRs (n=8)	Non-CKD (n=8)	*ES*	*P value*
IL-6				
Week 0	0.86 [0.64-1.38]	0.52 [0.37-0.64]	0.57	.021*
Week 4	0.86 [0.73-1.50]	0.40 [0.35-0.51]	0.66	.011*
Week 8	0.97 [0.48-1.66]	0.50 [0.33-0.70]	0.36	.161
6-months	0.90 [0.71-2.35]	0.50 [0.36-0.83]	0.51	.059
IL-10				
Week 0	0.51 [0.37-0.61]	0.44 [0.32-0.86]	0.13	.645
Week 4	0.51 [0.32-0.62]	0.36 [0.34-0.60]	0.11	.710
Week 8	0.55 [0.36-0.64]	0.41 [0.32-0.52]	0.31	.234
6-months	0.54 [0.42-0.64]	0.43 [0.36-0.74]	0.17	.549
TNF- α				
Week 0	3.82 [3.13-4.14]	2.49 [2.29-2.99]	0.70	.003*
Week 4	3.62 [3.32-3.99]	2.47 [2.01-2.74]	0.83	.001*
Week 8	3.79 [2.90-4.02]	2.50 [2.09-2.79]	0.73	.002*
6-months	3.76 [3.02-4.31]	2.62 [2.48-2.93]	0.58	.029*

Data presented as median and interquartile range for all groups * denotes significant difference between KTRs and non-CKD (P<.05)

3.3.2 Markers of cellular inflammation

Lymphocyte subsets (figure 7-10)

There were no differences for absolute number or proportion of CD3+, CD19+, CD56+, CD4+, CD8+ and CD45RO+ between or within groups over time. HIIT B had greater naive T cells than MICT at 3-months post training (P=.014, ES=-0.81). Regulatory T cells were greater in MICT than HIIT A at pre-training (P=.009, ES=-0.69).

Monocyte subsets

There were no differences for absolute number or proportion of monocyte subsets between or within groups over time (P>.05).

3.3.3 Markers of circulating inflammation

No differences were observed for IL-6, IL-10 or TNF-α between or within groups over time (P>.05).

4 DISCUSSION

The main aims of this manuscript was to discuss and then explore the interaction of immune and inflammatory cells in relation to exercise in KTRs. This manuscript comprised of two studies: MAP-KD; in which the main aim was to observe immune and inflammatory cells in stable KTRs and non-CKD controls over a longitudinal period of 6 months to provide comparative data for the PACE-KD trial; an 8 week exercise intervention study exploring HIIT and MICT in KTRs.

In MAP-KD, the main findings were that eGFR and T cell subsets were all higher in non-CKD controls than KTRs. TNF-α and IL-6 were greater in KTRs then non-CKD controls. Following 8-weeks of HIIT and MICT, there were no differences in immune and inflammatory cells in KTRs.

4.1 INFLAMMATION

In the present study, there was an increase in the percentage of CD4+ cells in healthy controls and an increased trend in CD8+ cells in KTRs, but a lack of difference in both groups over time. Previous literature has found no differences between healthy controls and KTRs in the percentage or absolute number of CD4+ and CD8+ T cells two and five years post transplantation (141). No difference between these populations could be a result of optimum immunosuppressive medication regimes. Immunosuppressive medication dosage is initially calculated on a mg/kg bodyweight basis, after a trial-and-error approach is accepted (49), the KTRs in the present study were transplanted 7 ± 5 years ago, and therefore it may be fair to suggest that that optimal dosages of immunosuppressants for this group have been obtained, it may be plausible to suggest that lack of variation over time in immune cell markers for KTRs may also be a result of optimised immunosuppressive medications.

Monocyte subsets percentages were not different between KTRs and non-CKD controls. Patients with ESRD have been shown to have high proportions of circulating non-classical monocytes (142). However, in patients 6-months post renal transplant, there have been observations of increases percent-

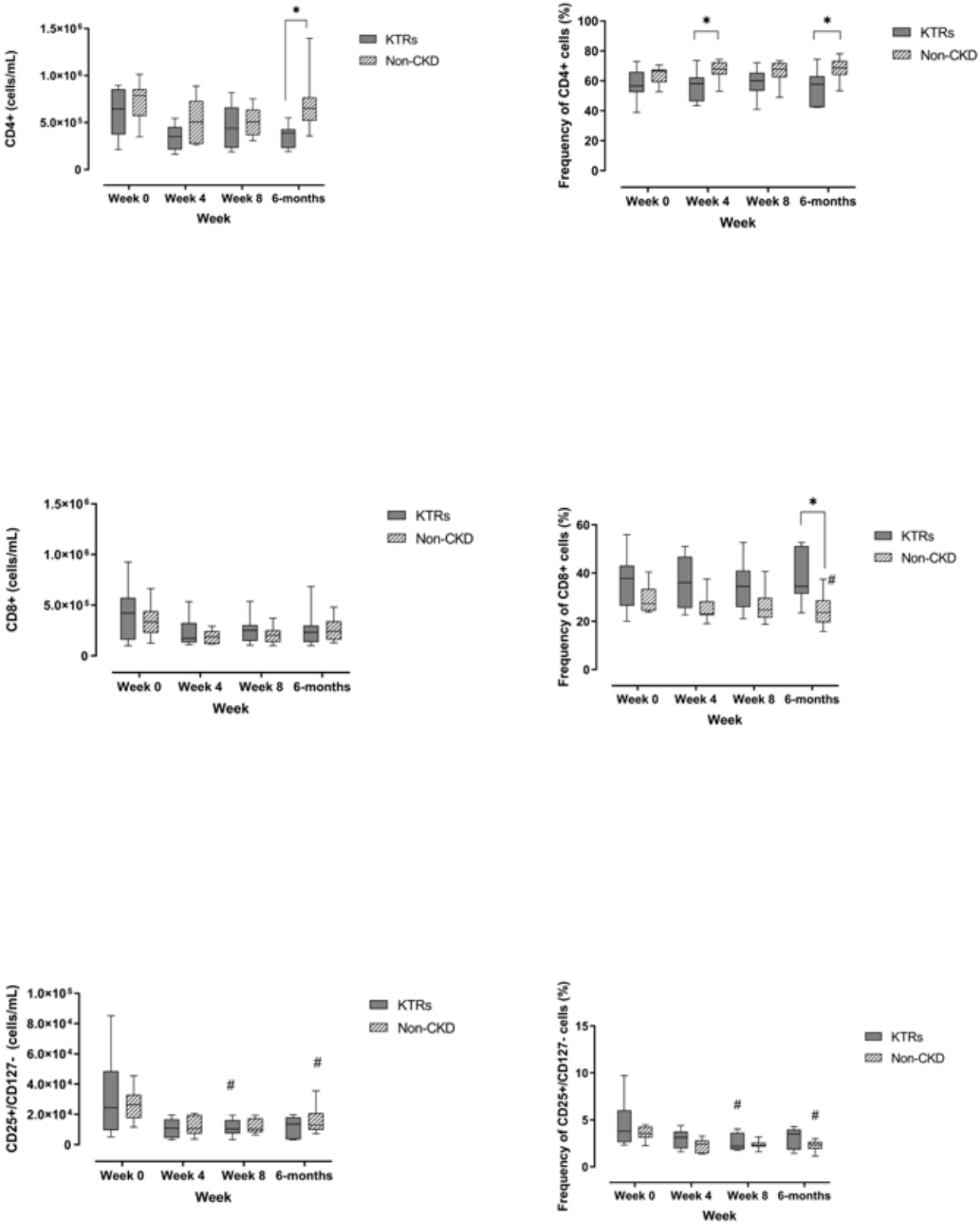

Figure 4. Cell numbers (cells/mL) and cell proportion (%) for CD4+, CD8+, CD25+/CD127- cells at week 0, week 4, week 8 and 6-months for KTRs and Non-CKD controls KTR; kidney transplant recipients, non-CKD; non-chronic kidney disease, non-normally distributed data presented as median and interquartile range. # Denotes significant difference from week 0 for non-CKD and KTRs

* Denotes a significant difference between groups

* Denotes a significant difference between groups, non-CKD greater KTRs

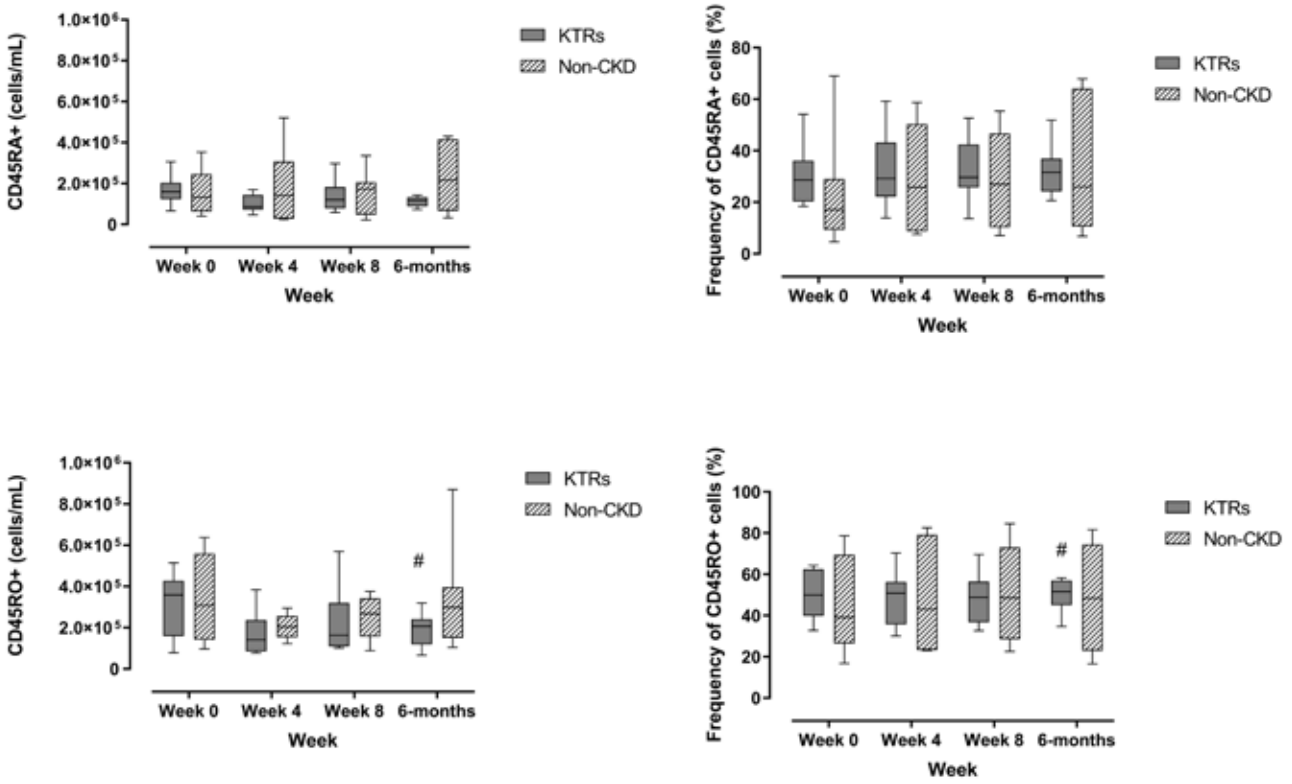

Figure 5. Cell numbers (cells/mL) and cell proportion (%) for CD45RA+, CD45RO+ cells at week 0, week 4, week 8 and 6-months for KTRs and Non-CKD controls KTR; kidney transplant recipients, non-CKD; non-chronic kidney disease, non-normally distributed data presented as median and interquartile range. # Denotes significant difference from week 0 for KTRs
* Denotes a significant difference between groups, non-CKD greater KTRs

ages of CD16+ monocytes compared to healthy controls, initiating an enhanced production of TNF- α, IFN-γ and IL-1β (143), and remaining elevated irrespective of immunosuppressive medications. The present study found no differences in monocyte subsets in stable transplant patients but found elevated concentrations of TNF- α and IL-6. Elevations in TNF-α and IL-6 have been associated with CVD, with reports showing that elevated cytokines is a predictor of death in dialysis patients (144). There have been suggestions that the increase in cytokines could be attributed to either specific renal diseases or other chronic diseases associated with transplantation (145). The KTRs included in this study had been post-transplant 7 ± 5 years, three of these patients suffered from diabetes mellitus, all suffered from hypertension, and one patient suffered from heart disease. These diseases have been associated with inflammation (146, 147). There has also been evidence that greater time post-transplant is associated with increased systemic inflammation and disease (148); however, it is up for discussion whether the inflammation facilitates traditional risk factors for CVD, or whether the CVD itself causes the chronic inflammation. The novelty in the present study findings are that the elevations in cytokine concentrations were independent to monocyte subsets. Further investigation is needed with in vitro analysis to assess cytokine production by monocytes in KTRs and non-CKD controls.

Regular exercise is suggested to be a protective mechanism of CV risk which may indirectly protect against vascular and systemic low-grade inflammation (12, 149). Previous data has reported an anti-inflammatory effect in CKD populations following moderate intensity exercise (121). Reductions in visceral fat mass and increased production of anti-inflammatory cytokines from contracting skeletal muscles are thought the be the mechanisms of the effect (14). During and following exercise, active skeletal muscle increases both cellular and circulating levels of IL-6. The transient rise in circulating IL-6 during exercise appears to be responsible for a subsequent rise in circulating levels of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1RA; (14)), however due to low sample size and lack of plasma samples for acute exercise bouts, we cannot be sure this is true to our patient group.

Our data did not report any changes in long term for circulating cytokines following the exercise intervention. However, higher concentrations of circulating IL-6 and TNF-α were reported in non-exercising transplant patients compared to non-CKD controls. Despite the intensity of the exercise, the study found no evidence for increased chronic inflammation, suggesting HIIT and MICT to not exacerbate inflammation.

A particular side effect of immunosuppressive medication is weight gain (150), given that stable transplant patients are on life-long medications (49) there is a likelihood of increased fat mass. The accumulation of body fat, is associated with increased all-cause mortality (151) and further metabolic syndromes, which is exacerbated through increases in pro-inflammatory adipokines such as TNF-α and IL-6 (10), thus developing a persistent state of low-grade systemic inflammation. Although the present study saw no changes in body fat and body mass following exercise training, there were no further increases in circulating cytokines following 8-weeks of exercise. However, anecdotal evidence did report patients to see a greater weight gain in the subsequent years following

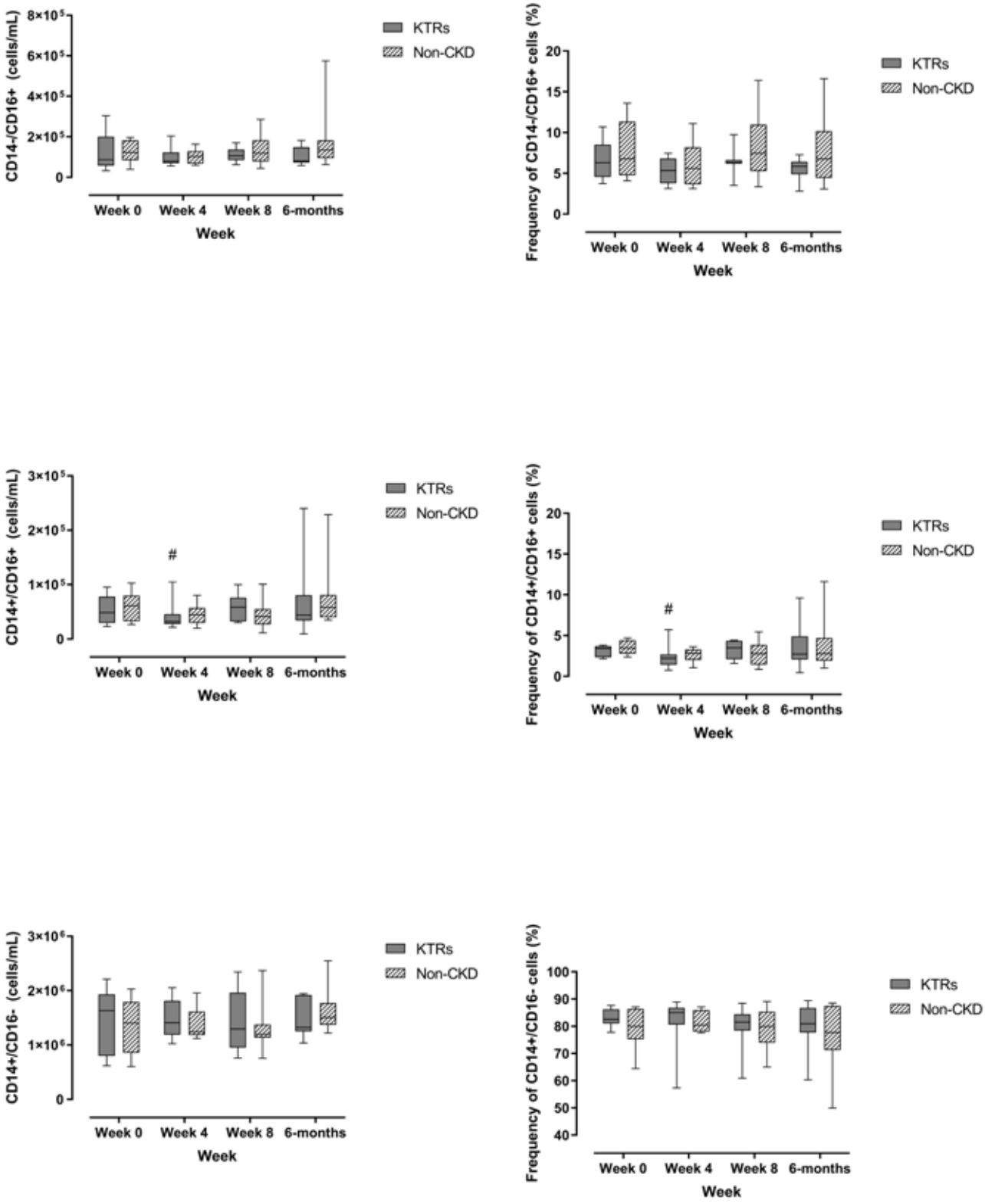

Figure 6. Cell numbers and cell proportion for monocyte subsets at week 0, week 4, week 8 and 6-months for KTRs and non-CKD. KTR; Kidney transplant recipients, non-CKD; non-chronic kidney disease, non-normally distributed data presented as median and interquartile range
# denotes significant difference from week 0 for KTRs

transplantation. It may be plausible that 8-weeks of training may not elicit changes in adipose tissue, but long-term exercise may elicit a reduction in adipose tissues resulting in reductions of circulating pro-inflammatory adipokines as seen in healthy populations.

Circulating immune cells only represent a small part of the immune system. Following exercise, the early inflammatory response is characterised with neutrophils entering the muscle followed by macrophages in regenerating muscle (89, 152). Macrophage-derived growth factors and cytokines are involved in myogenic precursor cell proliferation and differentiation. In animal studies, infusion of TNF-α, IL-1, and IL-6 has led to increased muscle protein breakdown and to muscle atrophy (153). An association between inflammation and muscle wasting has been reported in CKD patients (27). Further to this, reports of elevated mRNA expression of intramuscular IL-6, TNF-α, toll-like receptor-4 (TLR4) and myostatin, while NF-κB and p38 mitogen-activated protein kinase (MAPK) signalling is also upregulated in CKD populations (91, 154) likely resulting in intramuscular inflammation. Inflammation is a regular response to exercise, an 8-week resistance training study in CKD patients (82), reported increases in intramuscular IL-6, MCP-1, and TNF-α within 24 hours after a single exercise bout (155). It may be postulated that following the exercise, intracellular immune and inflammatory cells may be more active than circulating cells, resulting the minimal systemic changes seen in the present study.

4.1.1 Immune cells

Exercise immunology studies have reported transient time-dependant changes to phenotypic and functional capacity of lymphocytes in circulation in response to a single bout of exercise (16). For intense exercise, it is observed that the lymphocyte repertoire is increased during exercise which results in 'stimulation' of the immune system. However, hours following exercise, total lymphocyte numbers decrease to below pre-exercise levels proposing that exercise induces a short-term window of immune suppression. Exercise may deploy immune cells to peripheral tissues (156) , resulting in less mobilisation of immune cells within the circulation. A study by Kruger et al. (2008) found that T cells are redeployed in large numbers to peripheral tissues including the gut and lungs, and to the bone marrow following exercise by using fluorescent cell tracking (157).

4.2 IMPLICATIONS OF RESEARCH

Currently, unified exercise guidelines for KTRs do not exist. Exercise has been incorporated into the International Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines, which suggest some form of aerobic physical activity that is compatible with CV health and tolerance. However, there is a lack of detail for the type, frequency, intensity and duration of the exercise. Recommendations of at least 30 minutes of moderate intensity physical activity, 5 times a week have been suggested for weight management and improvements of CV health and QoL (68), however these studies look more at improving lifestyle factors for individuals with CKD stages 3-5 rather than KTRs (an immunocompromised population). The data in this manuscript suggests that recommending HIIT as a form of exercise is beneficial and can be incorporated into newly formed exercise guidelines for this population.

This project was developed with the help of exercising KTRs who were motivated to do intense exercise but were unsure as to whether it was immunologically safe for them. Given that physiological improvements in HIIT groups were observed without adverse negative effects on immune cells, it may be postulated that that HIIT is safe and tolerable for this population group.

Even though transplantation have been shown to reduce the risk of CV events (3), CVD still remains one of the major causes of mortality in KTRs (158) and outcomes remain poorer than in the general population (4). Chronic inflammation plays a major role in CVD risk (11, 40, 159, 160), and elevations in pro-inflammatory cytokines CRP, TNF-α, IL-6 and IL-1β have been demonstrated to contribute to systemic inflammation (20, 22, 27, 161, 162). In this study, elevations in IL-6 and TNF-α concentrations were reported in non-exercising KTRs compared to their non-CKD counterparts. Other data reports decrease in inflammatory cytokines in heart transplant patients following exercise (163). Given that exercise is suggested to be a therapeutic method to reduce CVD risk factors, and no increases in cytokines were observed following 8-weeks of exercise, HIIT can improve exercise capacity and physical function, but also for reducing CVD risk factors. However, further longitudinal investigations are needed to confirm this.

4.3 STRENGTHS

Given that exercise is becoming more popular with clinical populations for improvements in QoL, the safety and tolerance of different exercise programmes is a major concern. The study PACE-KD presented in this manuscript was designed in response to patient-generated research questions based on exercise performance enhancement and the interaction with immunosuppressive medication.

The flow cytometry panels used to assess the frequency of immune cells are novel panels devised for this study. The immune markers chosen to assess were markers that are suggesting altering from exercise interventions and relevant to kidney populations. The panels and analysis were developed to ensure that any changes in immune cell populations were a result of the exercise intervention rather than deviations of methodologies.

4.4 LIMITATIONS AND FUTURE DIRECTIONS

Some methodological limitations limit the conclusions that can be drawn from the above data. Whilst there were no differences for circulating immune cell subsets, we cannot be sure that these results are the same at the tissue level. Exercise is known to initiate muscle metabolism, therefore the use of muscle biopsies with the intention of immunostaining would generate practical data in addition to what has been presented in this manuscript.

Given that exercise can initiate an immunosuppressive effect in healthy populations (164), and the addition of immunosuppressive medications may initiate further immune suppression, this may result in a window for opportunistic infections. The data presenting in this study reports minimal changes to the frequency of immune cells following a single bout of acute exercise. Future studies may warrant the use of in vitro lymphocyte proliferation assay that would determine whether cells would be triggered to divide after exposure to a specific stimulus e.g. Phytohaemagglutinin P (PHA) or Concanavalin A (Con

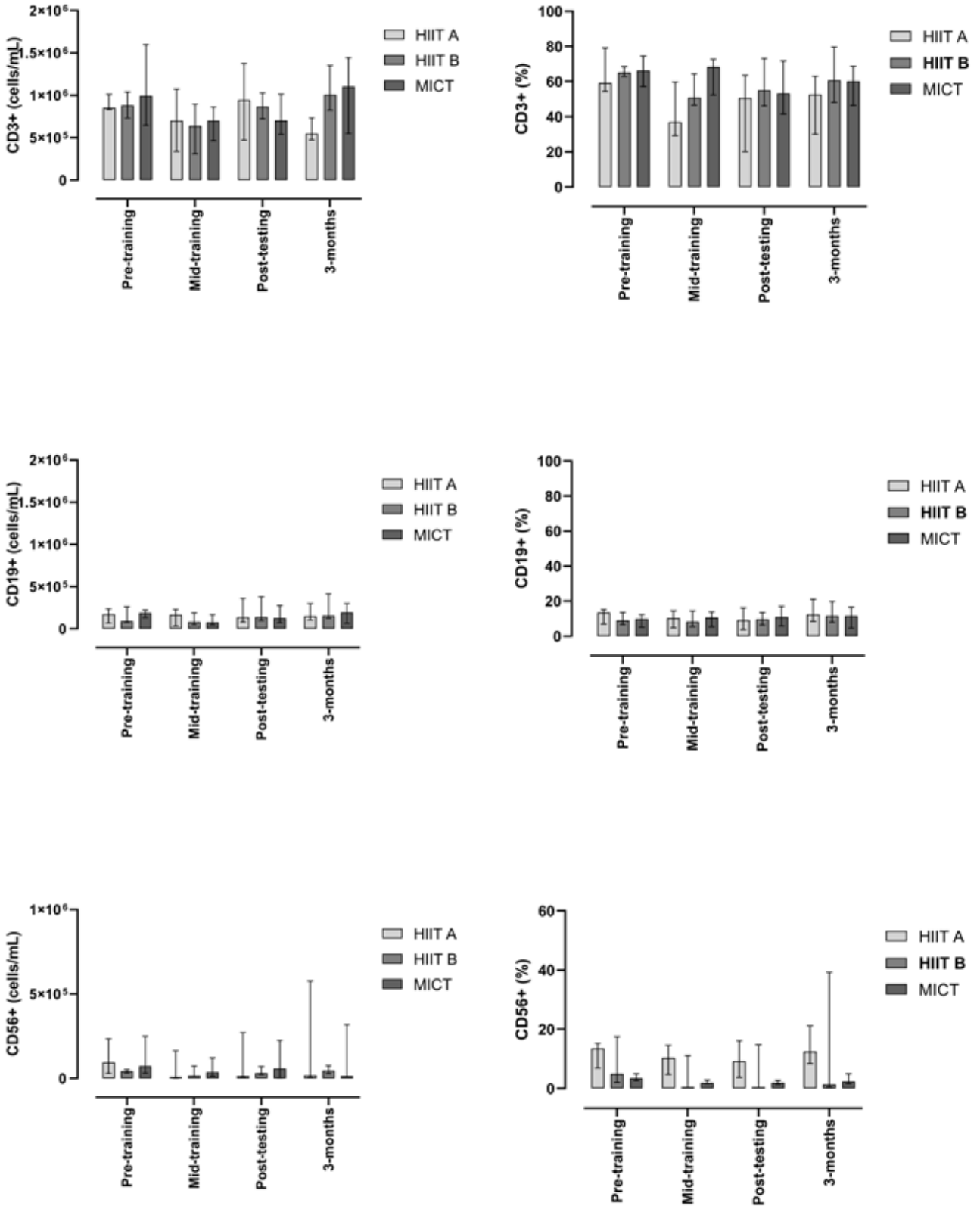

Figure 7. Cell numbers (cells/mL) and cell proportion (%) for CD3+, CD19+, CD56+ cells at pre-training, mid-training, post-training and 3-months post training. HIIT A; high intensity interval training A, HIIT B; high intensity interval training B, MICT; moderate intensity continuous training, non-normally distributed data presented as median and interquartile range

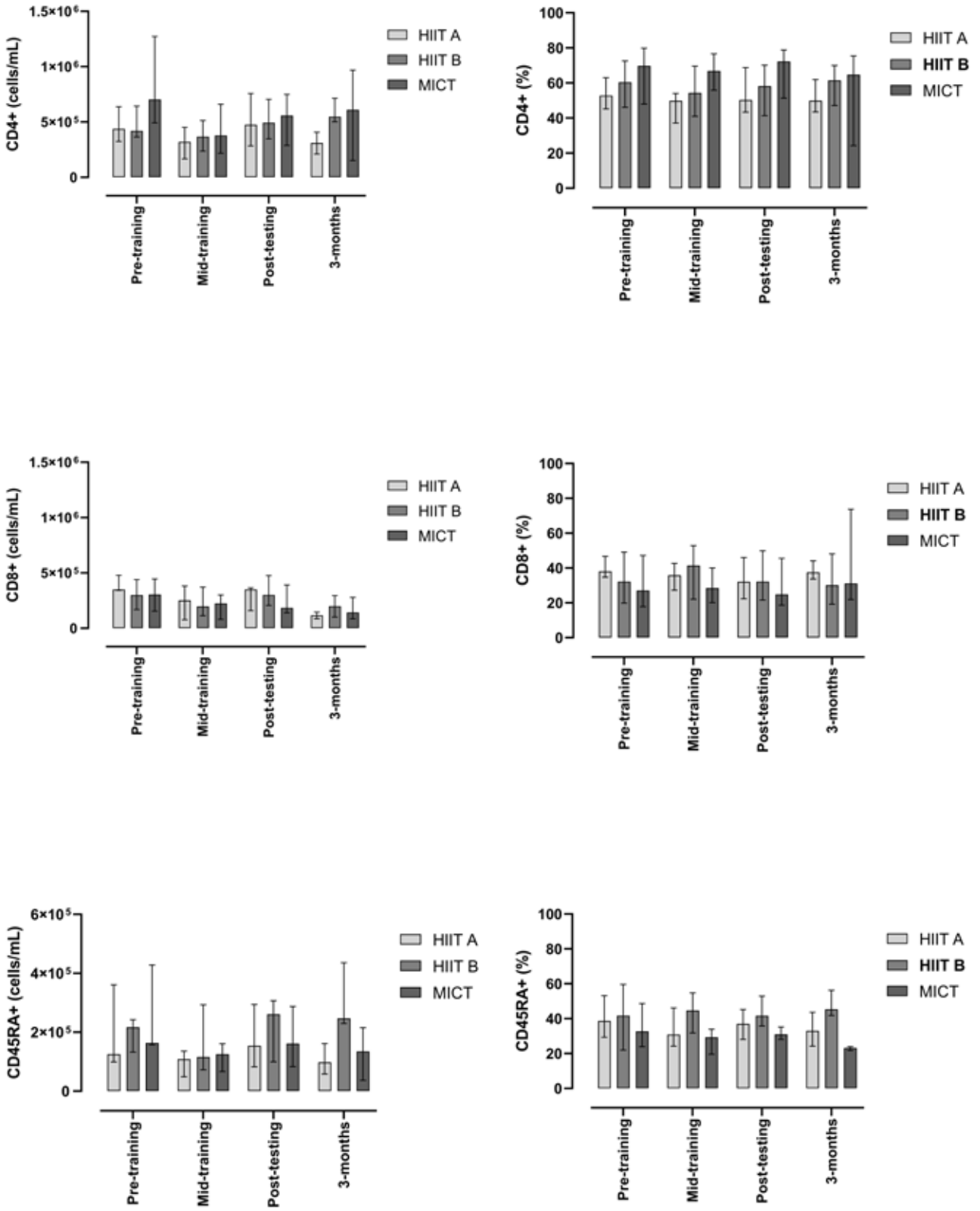

Figure 8. Cell numbers (cells/mL) and cell proportion (%) for CD4+, CD8+, CD45RA+ cells at pre-training, mid-training, post-training and 3-months post training. HIIT A; high intensity interval training A, HIIT B; high intensity interval training B, MICT; moderate intensity continuous training, non-normally distributed data presented as median and interquartile range

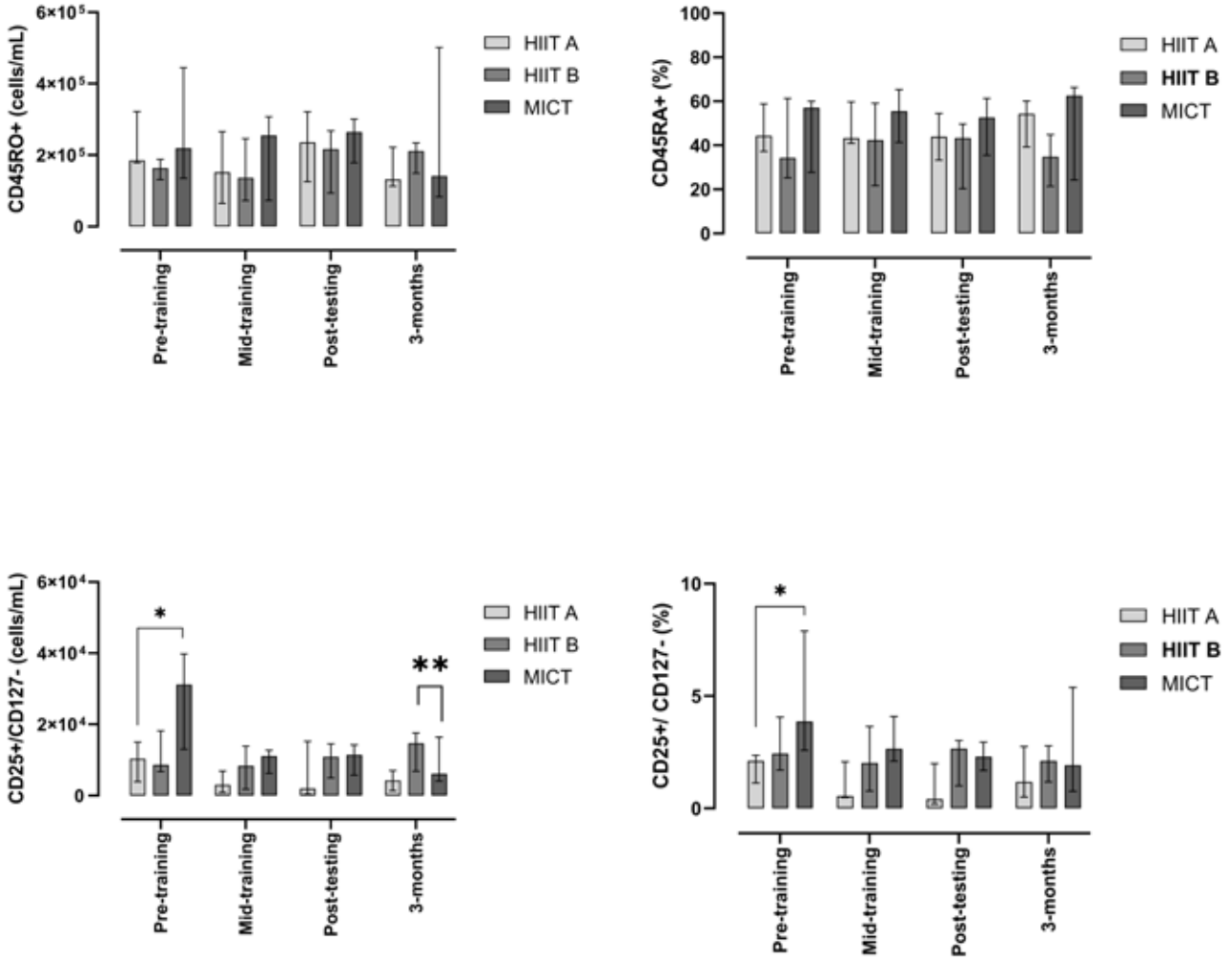

Figure 9. Cell numbers (cells/mL) and cell proportion (%) for CD45RO+, CD25+/CD127- cells at pre-training, mid-training, post-training and 3-months post training.
HIIT A; high intensity interval training A, HIIT B; high intensity interval training B, MICT; moderate intensity continuous training, non-normally distributed data presented as median and interquartile range
* denotes significant difference between groups at pre-training for CD25+ CD127- cells MICT greater than HIIT A
** denotes significant difference between groups at 3-months post training for CD25+ CD127- cells HIIT B greater than MICT

A). This method is quick and inexpensive however the assay would provide information about the health and response of the population of cells as a whole rather than individual cells. The alternative method that would allow information about individual cells is to label cells in culture with a fluorescent marker such as carboxyfluorescein succinamidyl ester (CFSE), which would dilute in each generation of daughter cells as they divide in response to a stimulus and could be measured via multi-colour flow cytometry. Assessing the proliferation and responsiveness of cells would add evidence to the interaction of exercise and immunosuppressive medications on immune cell subsets and may draw some conclusions on timing immunosuppressive medications around exercise, thus advancing the research area.

The HIIT protocols in this study have been used in previous clinical studies (109, 165), with beneficial effects. Following transplantation, exercise adherence is lowered after the first 5 years of transplant (150, 166). The present study found KTRs to attend exercise sessions either before or after work with some patients withdrawing from the study due to other commitments (manuscript under review elsewhere). Therefore, home-based exercise sessions may be more beneficial than hospital-based exercise programmes for greater exercise adherence and physiological benefit (167).

The data presented in this manuscript is from feasibility and pilot studies, therefore small sample size and inadequate detection of statistical changes are seen. However, data can be used to generate sample size calculations for larger trials. Future studies may also want to reduce the number of parameters detected/ time points to prevent spurious significant results.

Finally, exercise has been established as part of a multimodal therapeutic approach to cardiorespiratory fitness, muscle strength, physical function and improvement in quality of life (73). However, the type, duration, intensity of exercise remains obstacles

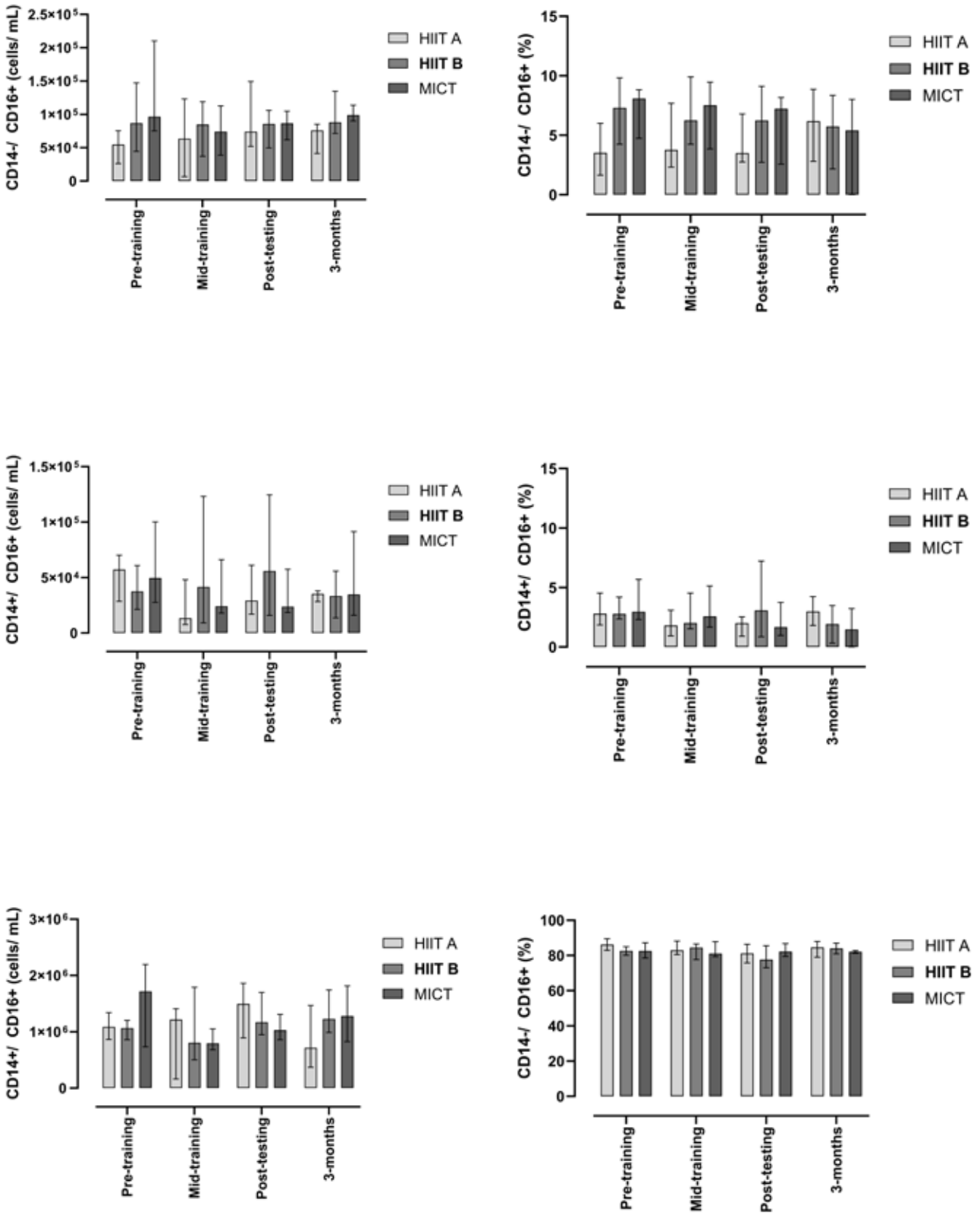

Figure 10. Cell numbers (cells/mL) and cell proportion (%) for monocyte subsets at pre-training, mid-training, post-training and 3-months post training.
HIIT A; high intensity interval training A, HIIT B; high intensity interval training B, MICT; moderate intensity continuous training, non-normally distributed data presented as median and interquartile range

in the assessment of measurable effects of exercise on inflammatory markers. This paper demonstrates that regular exercise may not have any negative effects on immune parameters in KTRs. As exercise helps to improve CV health, regular exercise should be encouraged following transplantation as it appears to have a physiological benefit. This manuscript is one of first forms of research to explore HIIT on immune cells of immunocompromised patients. Muscle metabolism studies are needed using non-exercising KTRs, exercising KTRs and healthy controls for the assessment of mitochondrial oxidative capacity and fluorescent immune cell trafficking within the muscle. This would allow researchers to investigate whether CKD and transplant populations are able to make physiological adaptations or initiate a training response. Inhibition of protein synthesis, increased protein catabolism and mitochondrial impairment leads to weakness and there is some evidence that immunosuppressant drugs may potentiate one another in their detrimental effects on skeletal muscle (168, 169).

Unanswered questions remain for the immunological consequences of HIIT and the interaction of immunosuppressive medications for the infection risk and graft rejection of this population. To investigate infection risk and graft rejection, firstly, a proliferative assay with an unspecific or specific stimulus in culture would identify how the cells response before and after the exercise interventions. Secondly, treating the cell cultures with an immunosuppressive reagent before and subsequently after exercise, would allow some conclusions to be drawn about the optimum timing of immunosuppressant regimes.

4.5 CONCLUDING REMARKS

CVD, infection, and malignancy are common in KTRs. Exercise can alter immunity. This study demonstrated that 8-weeks of HIIT and MICT programmes presented in this study did not cause any immediate adverse negative effects on immunity. KTRs can exercise at a range of high intensities without worrying about long-term alterations to immune parameters., however there must be caution when working at greater intensities than presented here.

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AUTHORSHIP:

A.C.S. and N.C.B. contributed to the research idea and study design. G.M.H., A.M.C., R.E.B., and D.G.D.N. contributed to the generation/collection of data. G.M.H. and A.M.C. were involved in data interpretation. A.M.C. and A.C.S. were involved in supervision. G.M.H. drafted the manuscript. Each author contributed important intellectual content during manuscript revision and accepts responsibility for the overall work.

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