The effects of short-term high intensity interval training compared to moderate-intensity continuous training on cardiometabolic health and inflammation in individuals at elevated risk of type 2 diabetes.

by

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Abstract

Chronic low-grade inflammation plays a key role in the development of insulin resistance and type 2 diabetes mellitus (T2DM). Exercise is known to reduce inflammation, although the underlying mechanisms responsible are not fully elucidated. One proposed mechanism is reduced expression of pro-inflammatory toll-like receptors (TLRs) on immune cells after exercise training. High intensity interval training (HIIT), when compared to traditional moderate-intensity continuous training (MICT), may result in superior cardiovascular and metabolic benefits but the impact of HIIT on inflammation has not been adequately studied. The aim of this thesis was to compare two weeks of HIIT to MICT for improving cardiometabolic risk factors and markers of inflammation, including TLRs, in previously inactive adults diagnosed with prediabetes.

Inactive adults with prediabetes (N=39, age 30 – 65 years) were recruited and randomly assigned to an exercise intervention involving 10 days of progressive HIIT (4-10 X 1-min @ ~85% peak heart rate [HR_{PEAK}] 1-min rest periods) or MICT (20-50 min @ ~60% HR_{PEAK}) matched for estimated external work. Before and ~48-72 hr after the final training session, participants completed a maximal oxygen uptake (VO_{2PEAK}) test and markers of inflammation and cardiometabolic health were measured.

The central hypotheses that HIIT would result in greater improvements in cardiometabolic health and cause a larger reduction in markers of inflammation were not supported. Both HIIT (1.8 ± 0.4 vs. 1.9 ± 0.4 L/min, pre vs. post) and MICT (1.8 ± 0.5 vs. 1.9 ± 0.5 L/min, pre vs. post) were equally effective at improving VO_{2PEAK} ($p<0.001$). TLR4 expression was reduced on lymphocytes and monocytes after both HIIT and MICT ($P<0.05$) and on neutrophils after MICT only ($p<0.01$). TLR2 on lymphocytes was also significantly reduced after both HIIT and MICT ($p<0.05$). Plasma inflammatory cytokines and insulin were unchanged after training in both groups but MICT led to a reduction in fasting plasma glucose ($P<0.05$, 5.9 ± 1.0 vs. 5.6 ± 1.0 mmol/l, pre vs. post).

This study further supports HIIT as a viable training option for previously inactive adults at elevated risk of T2DM. However the increased duration of exercise associated with more traditional MICT may provide a greater stimulus reducing neutrophil TLR4 and lowering fasting glucose when compared to HIIT.

Preface

The design of this research study was developed by Drs. Jonathan Little and Mary Jung. Testing and training of participants was coordinated by Jessica Bourne and myself. Analysis of blood markers was performed by Cody Durrer, Svetlana Simtchouk and myself. I was responsible for all statistical analysis and writing that is presented in this thesis. Ethics for this thesis was approved by UBC clinical research ethics board, H12-02268.

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Sections of this thesis have been presented at the UBCO Interdisciplinary Health Conference (March 2014), American College of Sports Medicine Annual Meeting (May 2014) and Exercise Physiologists of Western Canada Conference (July 2014). The study presented in this thesis is part of a larger research project that monitors adherence to exercise following the two-week intervention. The following manuscript has been published from this project: Jung, M. E., Bourne, J. E., Beauchamp, M. R., Robinson, E., & Little J. P. (2015) High-intensity interval training as an efficacious alternative to moderate-intensity continuous training for adults with pre diabetes. *Journal of Diabetes Research, 2015: 191595. http://dx.doi.org/10.1155/2015/191595.*

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ACSM	American College of Sports Medicine
ADA	American Diabetes Association
AMPK	5’ Adenosine Monophosphate Activated Protein Kinase
AS160	AKT Substrate 160
BMI	Body Mass Index
CD	Cluster of Differentiation
CGM	Continuous Glucose Monitor
CPR	C-Reactive Protein
CSEP	Canadian Society of Exercise Physiologist
CVD	Cardiovascular Disease
DEXA	Dual Energy X-ray Absorptiometry
FFA	Free Fatty Acid
GLUT4	Glucose Transporter 4
HbA1c	Glycated Hemoglobin
HIIT	High Intensity Interval Training
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HOMA-β	Homeostasis Model Assessment of β cell function
HR_{PEAK}	Peak Heart Rate
HSP	Heat Shock Protein
IKK	IκB Kinase
IL	Interleukin

IRS-1	Insulin Receptor Substrate – 1
JNK	c-Jun N-Terminal Kinases
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein 1
MICT	Moderate Intensity Continuous training
NEFA	Non Esterified Fatty Acids
NK	Natural Killer cells
OGTT	Oral Glucose Tolerance Test
PAI-1	Plasminogen Activator Inhibitor – 1
PAMPS	Pathogen Associated Molecular Patterns
PARQ+	Physical Activity Readiness Questionnaire
PBMC	Peripheral Blood Mononuclear Cells
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 α
PGN	Peptidoglycan
PI3K	Phosphoinositide 3 Kinase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxidative Species
SER	Serine
T2DM	Type 2 Diabetes Mellitus
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor – alpha
TYR	Tyrosine

VAT	Visceral Adipose Tissue
VO_{2PEAK}	Peak Volume of Oxygen Uptake
W_{PEAK}	Peak Watts

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1.0 Overview.

1.1 Type 2 diabetes mellitus.

Obesity, physical inactivity, and poor diet have all contributed to an increase in the worldwide prevalence of type 2 diabetes mellitus (T2DM). The magnitude of the problem that T2DM is placing on the healthcare system is highlighted by the decreasing age of diagnosis, to the point where children and adolescents are starting to develop T2DM (American Diabetes Association, 2000).

Alongside the increased prevalence of T2DM, there has been an increase in associated complications such as cardiovascular disease (CVD), stroke, some types of cancer, and Alzheimer's disease, which all contribute to rising healthcare costs related to T2DM (Yach, Stuckler, & Brownell, 2006). Canada has not been immune to this trend with the rate of T2DM increasing by 230% between 1998 and 2009. It is estimated that 3.7 million Canadians will be diagnosed with diabetes by 2018 with an estimated economic burden of $16.9 billion annually (Pelletier et al., 2012).

1.1.1 Pathophysiology of insulin resistance.

T2DM is a metabolic disease typically characterized by progressive worsening of insulin resistance and pancreatic beta (β) -cell insufficiency, ultimately leading to impaired glycemic regulation (Kahn & Porte, 1997). The major metabolic consequences are fasting and postprandial hyperglycemia. T2DM is typically diagnosed when fasting blood glucose is ≥7.0 mmol/L and/or two-hour post oral glucose tolerance test (OGTT) blood glucose is ≥11.1 mmol/L (Alberti, Kurt George

Matthew Mayer & Zimmet, 1998). A glycated hemoglobin (HbA1c) ≥6.5% (indicative of the degree of glucose control over the previous 2 – 3 months) can also be used to diagnose T2DM (Alberti, Kurt George Matthew Mayer & Zimmet, 1998). Although the diagnosis of T2DM is based on pre-defined blood glucose values, the development of T2DM can be viewed as a continuum of glycemic dysregulation that begins well before disease diagnosis. There are multiple studies that indicate the onset of T2DM pathophysiology can start up to 10 years prior to eventual diagnosis (Ferrannini et al., 2004; Tabák, Herder, Rathmann, Brunner, & Kivimäki, 2012).

Skeletal muscle, adipose tissue, and liver are the major insulin responsive tissues that form a feedback loop with the β-cells of the pancreas in order to control glucose uptake through insulin-mediated pathways (Kahn & Porte, 1997). In individuals with chronic nutrient overload and inadequate physical activity the balance between endogenous glucose production and glucose uptake into these insulin-dependent organs of the body is impaired (Scheen, 2003). The feedback loop between skeletal muscle, adipose tissue, and liver with pancreatic β-cells is initially able to increase insulin production in compensation (Kahn, Cooper, & Del Prato, 2014), which maintains glucose homeostasis in the interim. However, it is not a sustainable solution for insulin resistance and results in relative hyperinsulinemia (Defronzo, 2009). With no improvement in insulin sensitivity, β-cells experience increased oxidative and endoplasmic reticulum stress through constant activation, which in conjunction with glucolipotoxicity, results in β-cell apoptosis and eventual failure (Jurgens et al., 2011; Nolan, Damm, & Prentki, 2011; Poitout & Robertson, 2008). Thus, as insulin resistance and β-cell failure progress, the chronic

hyperglycemia that defines T2DM ensues.

Skeletal muscle is responsible for ~70-80% of the glucose disposal following ingestion of carbohydrates (DeFronzo & Tripathy, 2009; Ferrannini et al., 1988). In individuals with insulin resistance, there is a lack of glucose uptake into skeletal muscle contributing to the development of postprandial hyperglycemia. Individuals with T2DM also present with impaired insulin-mediated muscle glycogen synthesis (Macauley, Smith, Thelwall, Hollingsworth, & Taylor, 2015), which is most likely caused by lack of insulin-dependent glucose transport into the muscle (Shulman et al., 1990). Thus, improving skeletal muscle insulin resistance is regarded as a key target in the prevention and treatment of T2DM (DeFronzo & Tripathy, 2009).

Insulin resistance in the liver impairs the signal to inhibit glycogenolysis and gluconeogenesis, resulting in hyperglycemia. Despite this hyperglycemic state, the liver continues to produce glucose. This primarily contributes to the fasting hyperglycemia found in individuals diagnosed with T2DM (Defronzo, 2009). The inability of insulin to inhibit hepatic glucose production also contributes to postprandial hyperglycemia in individuals with, and at risk for, T2DM (Rizza, 2010).

Adipose tissue insulin resistance also contributes to hyperglycemia and exacerbates systemic insulin resistance by promoting ectopic fat deposition. Within adipose tissue insulin stimulates the conversion of glucose to triglycerides and increases lipoprotein lipase activity to stimulate triglyceride uptake from plasma (Ruan & Lodish, 2003). Insulin also acts to inhibit adipose tissue lipolysis and free fatty acid (FFA) release, promoting the uptake and storage of energy (Shulman, 2014). Thus, insulin resistance within adipose tissue leads to excessive lipolysis and

FFA release (Ruan & Lodish, 2003). The increase in circulating FFA is thought to drive some of development of insulin resistance in other tissues, specifically skeletal muscle and liver, as a build-up of fatty acid intermediates promotes lipotoxicity and impaired insulin signaling (Boden, Chen, Ruiz, White, & Rossetti, 1994; Schenk, Harber, Shrivastava, Burant, & Horowitz, 2009; Schenk & Horowitz, 2007; Shulman, 2014). Together, skeletal muscle, liver, and adipose tissue insulin resistance, intertwined with progressive β-cell dysfunction, are the main pathophysiological features contributing to the development of hyperglycemia and T2DM.

1.1.2 Prediabetes.

The term prediabetes has been developed relatively recently and identifies individuals who have an elevated fasting glucose, impaired glucose tolerance, and/or elevated HbA1c that is not yet severe enough to be classified as T2DM (Alberti, Kurt George Matthew Mayer & Zimmet, 1998). Prediabetes is a state of elevated risk for the development of T2DM and CVD (Malik et al., 2004). Although diagnostic criteria for prediabetes can vary, it is defined by the American Diabetes Association (ADA) as fasting blood glucose of 5.6 - 6.9 mmol/L and/or a two-hour post OGTT blood glucose of 7.8 - 11 mmol/L and/or an HbA1c of 5.7 - 6.4%. Not all individuals with prediabetes will develop T2DM, but ~50% of the estimated 5.7 million Canadians with prediabetes will develop T2DM in their lifetime (Knowler et al., 2002; Pelletier et al., 2012).

Similar to T2DM, prediabetes is characterized by insulin resistance and β-cell insufficiency and this pathophysiology begins prior to overt hyperglycemia and/or observable changes in glucose tolerance (Defronzo, 2009). Initially, increased

insulin secretion by β-cells is able to maintain fasting and postprandial glucose concentrations. However, this progresses to a state of decompensation whereby clinically significant increases in fasting and/or postprandial glucose occur, prompting a diagnosis of prediabetes (Tabák et al., 2012). Furthermore, many individuals with prediabetes already have sustained damage to various organs and longitudinal studies have established increased risk of CVD, stroke and Alzheimer's disease in individuals who have impaired glucose tolerance but are not diagnosed with T2DM (Tabák et al., 2012; Vermeer et al., 2006; Weyer, Tataranni, Bogardus, & Pratley, 2001).

Individuals with prediabetes are an excellent target group for lifestyle interventions because at this stage, insulin resistance may be reversible (Tuomilehto et al., 2001). Indeed, landmark clinical trials have shown that increased physical activity and modest weight loss through diet are effective in improving glucose control and preventing the onset of T2DM (Knowler et al., 2002; Li et al., 2008; Tuomilehto et al., 2001).

1.2 Inflammation.

The immune system employs a complex arrangement of barriers, both physical and cellular, defending the body against bacteria, viruses and pathogens. The cellular components of the immune system can recognize, attack and destroy foreign cells and organisms to maintain homeostasis and health (Kindt, Goldsby, Osborne, & Kuby, 2007). When the immune system works efficiently the harmful stimuli is removed and healing begins. However, prolonged activation of the immune

system can result in negative consequences such as the absence of healing and destruction of healthy tissues (Kindt et al., 2007).

White blood cells, which can be differentiated into multiple cell types, each have a unique role within the immune system. These cell types can be further divided into two arms of the immune system, innate immunity and adaptive immunity (Kindt et al., 2007). The innate immune system is the first line of defense and typically acts over minutes to hours. This system deploys the same non-specific predefined response to a pathogen, using a small number of pre-programed immune responses (Gleeson, Bishop, & Walsh, 2013). The adaptive immune response has a diverse number of receptors and produces antibodies that are specific to detected antigens and viruses. In addition adaptive immunity has an immunologic memory, allowing it to eliminate the pathogen more effectively during repeat infections. An adaptive immune response has a longer response time compared to the innate immune response, taking days to be initiated and lasting longer (Kindt et al., 2007). There is some research to suggest that the adaptive immune response has a small role in the development of obesity related chronic low-grade inflammation (Nishimura et al., 2009). However, in line with the majority of scientific evidence (Mathis & Shoelson, 2011), the role of the innate immune system in chronic low-grade inflammation will be the focus of this thesis.

1.2.1 Innate immune system.

The innate immune system is comprised of physical barriers (e.g., skin) and a myriad of cell types. The two main cell groups involved in this system are lymphocytes (predominately natural killer cells) and phagocytes (neutrophils and

monocytes) (Kindt et al., 2007). Each cell type performs certain functions and together form an effective immune response. Immunophenotyping can be performed through identification of surface markers that are specific to each cell type. Cluster of differentiation (CD) is the nomenclature given to the unique surface molecules that allow certain leukocytes and their subtypes to be identified.

1.2.1.1 Neutrophils.

Neutrophils are the most prolific leukocyte in circulation making up 50-70% of the population and are identified by the surface expression of certain markers, including CD15. Neutrophils are fast acting cells and are the first to migrate to the site of infection and initiate the immune response (Kindt et al., 2007). As a phagocytic cell, neutrophils engulf material at the site of infection in order to stimulate the immune response and to use digestive enzymes within the neutrophil to destroy it. Neutrophils are also responsible for an oxidative or respiratory burst, which releases reactive oxidative species (ROS) and reactive nitrogen species (RNS) to aid in the immune response (Fialkow, Wang, & Downey, 2007).

1.2.1.2 Monocytes.

Monocytes are also phagocytic cells, making up 5 – 15% of the total circulating leukocyte population and can be identified by their CD14 surface antigen. Immature monocytes circulate in the blood stream for up to 8 hours, after which they can migrate into various tissues, where they mature and become macrophages (Kindt et al., 2007). The important role of macrophages in adipose tissue inflammation is discussed below in Section 1.2.3. Monocytes use similar antipathogenic mechanisms to neutrophils, with the ability to phagocytose foreign

material and release ROS and RNS (Gleeson et al., 2013). Additionally monocytes release inflammatory mediators and cytokines, and have an important role in connecting the innate to the adaptive immune response (Kindt et al., 2007).

Despite monocytes making up a small percentage of the white blood cell population, expression of cell surface markers and cytokine production by monocytes have been the most widely measured immune response in exercise immunology research (Child, Leggate, & Gleeson, 2013; Simpson et al., 2009; Timmerman, Flynn, Coen, Markofski, & Pence, 2008). This may be due to the widely used peripheral blood mononuclear cell (PBMC) isolation technique, in which monocytes can be separated from whole blood for downstream applications. Another possible reason for the focus on monocytes may be because these white blood cells infiltrate tissues (e.g., adipose) to become macrophages, which are the cells believed to be primarily responsible for driving chronic low-grade inflammation in metabolic disease (see below in Section 1.2.3; (Gregor & Hotamisligil, 2007).

1.2.1.3 Lymphocytes.

Lymphocytes are a group of white blood cells that originate from lymphoid progenitor cells and are comprised of natural killer cells (NK), T cells and B cells. T and B lymphocytes are predominately active in the adaptive immune response, displaying specificity and immunologic memory to antigens using antigen binding cell surface receptors (Kindt et al., 2007). However NK cells, which are granulocytes like neutrophils and monocytes, play a role in the innate immune response. NK cells form the initial defense attack against viral infections, killing infected cells and

keeping the virus contained until the adaptive immune response is employed (Cerwenka & Lanier, 2001).

1.2.2 Insulin resistance and inflammation.

It is now well established that insulin resistance underlying T2DM and prediabetes is associated with a state of chronic low-grade inflammation (Mathis & Shoelson, 2011). This chronic low-grade inflammation appears to be linked to an exacerbated innate immune response as a result of metabolic dysfunction and/or nutrient overload and therefore has been labeled “meta-inflammation” (Hotamisligil, 2006). Increased markers of inflammation have been shown in circulating immune cells, adipose tissue, liver, skeletal muscle, and endothelial cells in insulin resistant humans and in animal models (Bruun, Helge, Richelsen, & Stallknecht, 2006; Dandona et al., 1998; Fontana, Eagon, Trujillo, Scherer, & Klein, 2007; Park et al., 2010; Rösen et al., 2001). Chronic low-grade inflammation in metabolic disease is typically characterized by an increase in basal levels of circulating pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF) – α (Hotamisligil & Spiegelman, 1994) and interleukin (IL) -6 (Krogh-Madsen, Plomgaard, Moller, Mittendorfer, & Pedersen, 2006), acute-phase reactants [e.g., C-reactive protein (Gleeson et al., 2011)], and adipokines [e.g., plasminogen activator inhibitor-1 (PAI-1) and leptin (Fontana et al., 2007)]. This basal increase in circulating inflammatory molecules is implicated not only in the pathogenesis of insulin resistance and T2DM (Gregor & Hotamisligil, 2007; Lumeng & Saltiel, 2011) but also in the development of CVD and atherosclerosis (Curtiss & Tobias, 2009). While the proximal cause of chronic low-grade inflammation has not yet been fully elucidated, it is known that metabolic

disruptions associated with insulin resistance (i.e., hyperglycemia and elevated FFA) can directly trigger innate immune responses through a number of pathways.

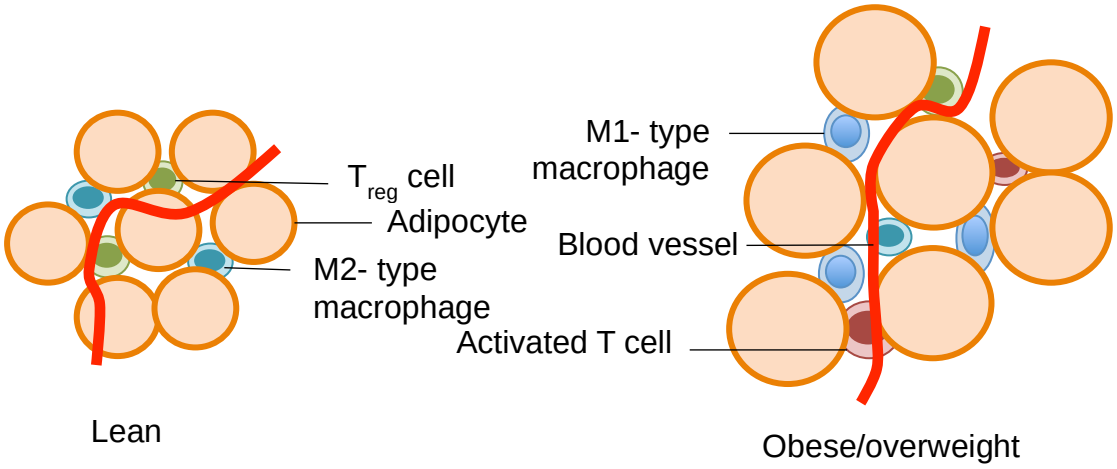
1.2.3 Adipose tissue inflammation.

Adipose tissue was once thought of as an inert organ, with the primary function of storing excess energy. However it is now evident that adipocytes have both immune (Weisberg et al., 2003) and endocrine functions (Zhang et al., 1994). Original research into the relationship between inflammation and metabolic dysfunction found that adipocytes in obese animals express more TNF-α (Hotamisligil & Spiegelman, 1994). Since this finding the role of adipose tissue contributing to chronic low-grade inflammation in obesity has been the focus of much research (reviewed by (Fantuzzi, 2005).

Studies have shown that obesity and T2DM are associated with an increased infiltration of pro-inflammatory immune cells such as neutrophils, “classically-activated” M1- type macrophages, and activated T cells within adipose tissue (Weisberg et al., 2003; Xu et al., 2003). The migration of immune cells into adipose tissue increases the release of pro-inflammatory cytokines (often referred to collectively as “adipokines”). In turn, chemokines such as monocyte chemoattractant protein-1 (MCP-1) are released from adipose to attract more monocytes, further increasing macrophage infiltration to create a self-perpetuating cycle of inflammation (Christiansen, Richelsen, & Bruun, 2005).

Within the adipose tissue of lean individuals there are a number of anti-inflammatory immune cells, including “alternatively-activated” M2- type macrophages and CD4+ regulatory T cells. The increase in adipose tissue mass as

a result of positive energy balance (nutrient overload and/or reduced physical activity) appears to result in a transformation of anti-inflammatory M2-type macrophages into pro-inflammatory M1-type macrophages (Lumeng, Bodzin, & Saltiel, 2007). Coupled with chemotactic attraction of circulating monocytes, adipose tissue becomes a site of chronic inflammation. Changes to adipose tissue under conditions of nutrient overload and lack of physical activity are seen in Figure 1.



Adapted from: (Gleeson et al., 2011)

Figure 1. **Changes in adipose tissue under conditions of chronic nutrient overload and lack of physical activity**. Anti-inflammatory immune cells within lean adipose tissue include M2-type macrophages and regulatory T (T_{reg}) cells. Increased infiltration of pro-inflammatory immune cells (M1-type macrophages and activated T cells) in obese/overweight adipose tissue with associated increased release of pro-inflammatory adipokines into the circulation contributes to systemic chronic low-grade inflammation.

In addition to inflammatory cell infiltration of adipose tissue, adipose tissue hypoxia may also contribute to increased inflammation (Trayhurn, Wang, & Wood, 2008). As adipose tissue expands, clusters of adipoctyes that do not have sufficient vascular supply can become hypoxic inducing the expression of hypoxia-inducible factor 1α and 1β and inflammatory proteins to further promote adipose tissue inflammation (Pasarica et al., 2009). The release of pro-inflammatory cytokines and adipokines contributes not only chronic low-grade inflammation but also the development of insulin resistance directly within adipose tissue and systemically.

1.2.4 Hyperglycemia and inflammation.

Acute excursions into hyperglycemia in humans can directly cause elevations in a number of circulating pro-inflammatory cytokines (e.g., TNF-α, IL-6 and IL-18). Hyperglycemia-induced inflammation can last for several hours in individuals with

impaired glucose tolerance (Esposito et al., 2002). Increases in cytokines are inhibited by antioxidant infusion, suggesting that oxidative stress mediates the induction of cytokines with hyperglycemia (Esposito et al., 2002). This has been confirmed *in vitro* where an increase in leukocyte ROS production can be detected upon exposure to hyperglycemic culture conditions (Mohanty et al., 2000). If acute increases in plasma glucose can induce inflammation then the chronic hyperglycemia experienced by individuals with prediabetes and T2DM would be hypothesized to perpetuate inflammation in the basal state as well as in response to postprandial glucose spikes. Indeed, individuals with elevated glucose have higher basal concentrations of circulating markers of inflammation, including C reactive protein (CRP), circulating pro-inflammatory cytokines, and PAI-1, all of which are linked to an increased risk of T2DM (Dandona, Aljada, & Bandyopadhyay, 2004 Pradhan, Manson, Rifai, Buring, & Ridker, 2001) and CVD (Sattar et al., 2003)

Hyperglycemia not only impacts circulating cytokines but also has direct pro-inflammatory effects at the cellular level. Immune cell phenotype can be altered by hyperglycemia, through changes in surface protein expression that increase the inflammatory nature of leukocytes. High glucose *in vitro* (Dasu, Devaraj, Zhao, Hwang, & Jialal, 2008) and hyperglycemia *in vivo* (Dasu, Devaraj, Park, & Jialal, 2010) are linked to elevated expression of toll-like receptors (TLRs), which are key cellular markers of innate immune activation. The activation of immune cells can be triggered under high glucose culture conditions that augment lipopolysaccharide (LPS)-induced pro-inflammatory cytokine release (Markofski et al., 2014; Morohoshi, Fujisawa, Uchimura, & Numano, 1996).

Even though the association between hyperglycemia and chronic low-grade inflammation has been confirmed in multiple studies using multiple markers of inflammation, the proximal cause of T2DM-related inflammation *in vivo* remains unknown. Hyperglycemia can cause increases in inflammation; however, increased inflammation is also known to result in insulin resistance, contributing to hyperglycemia. Thus, the exact role of meta-inflammation in the progression of T2DM is still unresolved; and it is unknown if it is the inflammation or the hyperglycemia that develops first.

1.2.5 Mechanisms linking chronic low-grade inflammation with insulin resistance.

While we might not know whether inflammation or hyperglycemia develop first, the concept of meta-inflammation is important in understanding the pathogenesis of T2DM because it suggests that our bodies are hardwired to detect, and react to, T2DM-related metabolic disturbances. Hyperglycemia- and hyperlipidemia-induced inflammation are of particular importance to the development and progression of T2DM because it implies the existence of a vicious cycle composed of insulin resistance, hyperglycemia/hyperlipidemia and inflammation (Figure 2). While the initial stimuli for this continuous cycle between chronic low-grade inflammation and metabolic disturbances remains unknown, there are a number of known mechanisms through which inflammation leads to insulin resistance.

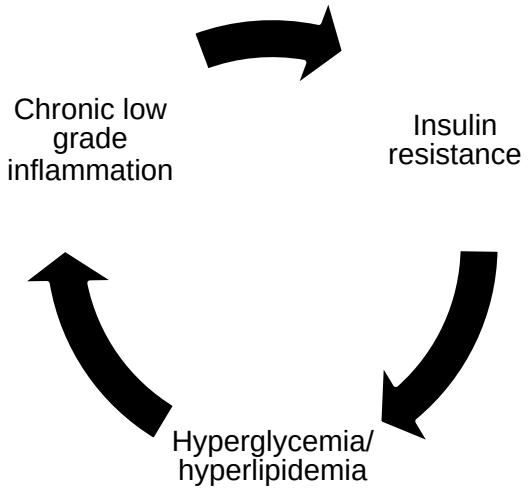
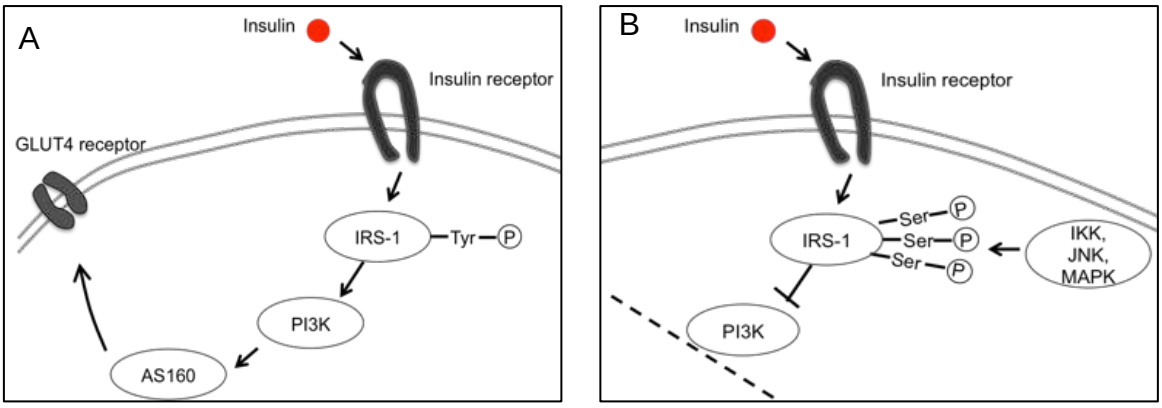


Figure 2. **Proposed vicious cycle between inflammation, insulin resistance, and metabolic dysfunction**. Hyperglycemia and hyperlipidemia as a result of insulin resistance can lead to chronic low-grade inflammation and the subsequent exacerbation of insulin resistance, which would result in further elevation of hyperglycemia and hyperlipidemia.

1.2.5.1 Activation of inflammatory signaling pathways.

Chronic low-grade inflammation results in the activation of a number of innate immune system pathways. Various kinases, including inhibitor of kappa B kinase (IKK) (Dandona et al., 2004), C-jun N-terminal kinase (JNK) (Solinas et al., 2007), and Mitogen activated protein kinases (MAPKs) (Gogg, Smith, & Jansson, 2009), which are activated as a result of inflammatory stimuli, can directly interfere with insulin signaling. This occurs because pro-inflammatory kinases - such as IKK, JNK and MAPKs - phosphorylate serine residues on the insulin receptor substrate 1 (IRS1) molecule, which inhibits insulin-mediated tyrosine phosphorylation and activation of IRS1. This process ultimately leads to a reduction in insulin signaling and impairs glucose transporter type 4 (GLUT4) trafficking to the cell membrane (Paz et al., 1997). As a consequence, heightened inflammation directly impairs

glucose uptake and contributes to hyperglycemia. The hypothesized interaction between inflammatory signaling and insulin signaling is depicted in Figure 3.



Adapted from: (Shulman, 2014)

Figure 3. **Molecular mechanism(s) by which inflammation can induce insulin resistance and glucose intolerance in muscle and liver cells**. Panel A: Simplified insulin signaling transduction pathway, whereby tyrosine phosphorylation of IRS-1 triggers GLUT4 translocation to the cell membrane via IRS-1, PI3K, and AS160. Panel B: Pro inflammatory kinases inhibit insulin signaling through serine phosphorylation of IRS-1, thus blocking GLUT4 translocation. *IRS-1, Insulin Receptor Substrate -1; PI3K, Phosphoinositide 3 kinase; AS160, Akt Substrate 160; GLUT4, Glucose transporter type 4; Tyr, Tyrosine; Ser, Serine; IKK, Inhibitor of kappa B kinase; JNK, c-Jun N-terminal kinases; MAPK, Mitogen-activated protein kinase*

Pro-inflammatory cytokines are directly linked with insulin resistance as they can activate inflammatory kinases, further impairing insulin signaling through the mechanism proposed in Figure 3 (Hotamisligil, Budavari, Murray, & Spiegelman, 1994). Further support that inflammatory signaling impairs insulin action is provided by studies in humans where infusion of pro-inflammatory cytokines can directly cause muscle insulin resistance (Plomgaard et al., 2005).

1.2.5.2 Toll-like receptors.

Another pathway through which inflammation may induce insulin resistance is through the activation of TLRs. TLRs are conserved transmembrane receptors expressed on numerous tissues, particularly immune cells (e.g., monocytes, neutrophils and leukocytes). TLRs recognize pathogen associated molecular patterns (PAMPs), which results in the activation of the innate immune response (Akira & Shizuo, 2003). The two main TLRs involved in chronic low-grade inflammation associated with disordered metabolism are TLR2 and TLR4. TLR2 primarily detects peptidoglycans of gram-positive bacteria while the classic ligand for TLR4 is LPS from gram-negative bacteria. Neutrophils, monocytes and lymphocytes all express TLR2 and TLR4 and can initiate innate immune responses following activation by a variety of pathogens. The activation of this immune response results in the up-regulation of pro-inflammatory cytokines, mobilization of chemokine receptors and increased T cell activation (Kaisho & Akira, 2006).

As discussed earlier, exposing human monocytes to hyperglycemic conditions *in vitro* can promote an increase in TLR2 and TLR4 expression (Dasu et al., 2008). This research was further supported by *in vivo* data showing increased TLR2 and TLR4 expression on monocytes in newly diagnosed T2DM patients compared to normoglycemic control subjects (Dasu et al., 2010). Not only is there increased TLR expression in individuals with hyperglycemia and T2DM but also increased circulating TLR2 and 4 agonists, for example LPS and FFA (Flynn & McFarlin, 2006).

Furthermore, individuals with obesity have been shown to have higher systemic concentrations of LPS compared to lean controls, possibly due to LPS entering the systemic circulation from a leaky gut. This could serve to promote inflammation through activation of TLR4 on circulating immune cells (Shi et al., 2006).

Non esterified fatty acids (NEFA; also known as FFA) are thought to play a role in contributing to insulin resistance and chronic inflammation. FFAs may act as TLR agonists (Dasu & Jialal, 2011) and infusion of FFAs has been shown to promote inflammation and impair insulin mediated glucose uptake (Itani, Ruderman, Schmieder, & Boden, 2002).

When increased TLR expression and activation is combined it likely perpetuates chronic low-grade inflammation and insulin resistance through increased pro-inflammatory signaling and cytokine release. This could increase intracellular activation of IKK and JNK in insulin target tissues, which, in turn, inhibits insulin signaling through serine phosphorylation of IRS-1 (Paz et al., 1997) (see Figure 3).

Further support for TLR involvement in insulin resistance comes from transgenic animal studies, where deletion or disruption of TLR2 (Ehses et al., 2010) and TLR4 (Tsukumo et al., 2007) have been shown to protect mice from insulin resistance induced by a high-fat diet. In addition to insulin resistance, increased TLR4 expression and activity on monocytes has been implicated in the development of atherosclerosis (Dasu et al., 2010). In line with the insulin resistance data, mice lacking TLR4 are protected against atherosclerosis (Michelsen et al., 2004) even

when fed a high-fat diet. Although there is much research to support a relationship between increased monocyte TLR expression and insulin resistance (Dasu et al., 2008; Flynn, McFarlin, Phillips, Stewart, & Timmerman, 2003; Timmerman et al., 2008), data on TLR expression on other immune cells (neutrophils and lymphocytes) and insulin resistance requires further investigation.

1.3 Insulin resistance and exercise.

Exercise promotes systemic health benefits through a variety of mechanisms including reducing excess adipose tissue and improving metabolic and cardiovascular health. Exercise is a potent stimulus for increasing insulin sensitivity, improving glucose control, and reducing the risk of T2DM (Boule et al., 2005; Cartee et al., 1989; Garcia-Roves et al., 2003). In individuals already diagnosed with T2DM, lifestyle interventions employing both exercise and diet modifications have been shown to be equal, or even more effective, compared to pharmaceutical approaches for improving glucose control and reducing the risk of developing complications associated with T2DM (Anderssen, Carroll, Urdal, & Holme, 2007; Boule et al., 2005; Orchard et al., 2005).

The importance of exercise for the treatment and maintenance of T2DM is reflected in the ADA recommendations (Colberg et al., 2010), which are in line with the American College of Sports Medicine (ACSM) guidelines and Canadian Society of Exercise Physiology (CSEP) recommendations. Both agencies recommend 150 minutes per week of moderate to vigorous intensity aerobic activity, most commonly prescribed as brisk walking, in bouts that are no more than two days apart.

Resistance training is also recommended on 2-3 days per week (Colberg et al., 2010). Despite substantial evidence on the benefits of exercise for improving insulin sensitivity and reducing the risk of T2DM, the optimal exercise therapy is still not well defined.

1.3.1 Mechanisms through which exercise reduces insulin resistance.

Exercise and physical activity can decrease of risk of developing T2DM as well as improve insulin resistance through a number of mechanisms. Exercise increases the metabolic requirement, thus more glucose as a substrate is converted to ATP (Goodyear & Kahn, 1998). Many of the benefits of exercise for improving insulin resistance are thought to be mediated at the level of the exercising skeletal muscle. To assist the increased demand for ATP, exercise (or muscle contraction) is able to mediate glucose transport into skeletal muscle via GLUT4 transporters (Douen et al., 1989; Fushiki, Wells, Tapscott, & Dohm, 1989). Exercise increases translocation of GLUT4 to the sarcolemma by a mechanism that is independent of insulin (Goodyear et al., 1990). Thus in individuals who are insulin resistant, exercise still results in an increase in muscle glucose uptake.

Exercise-induced muscle glycogen depletion is a stimulus for increased glucose uptake (Cartee et al., 1989) and increased rate of muscle glycogen synthesis (Perseghin et al., 1996) in the post-exercise recovery period. The end result is enhanced insulin signaling, decreased insulin resistance, and reduced hyperglycemia (Friedrichsen, Mortensen, Pehmøller, Birk, & Wojtaszewski, 2013). The underlying cellular and molecular mechanisms of exercise-induced increases in insulin sensitivity have not been fully elucidated but activation of skeletal muscle 5'-

AMP-activated protein kinase (AMPK) appears to be critical (Friedrichsen et al., 2013). A number of exercise-induced changes related to lipid accumulation and oxidation also appear to contribute to improvements in insulin resistance. First, changes in circulating FFA decrease the risk of developing T2DM and limit the impact of FFA on immune cells and insulin signaling (Shi et al., 2006). Second, decreased lipid deposits and increased fat oxidation within skeletal muscle with exercise training are also mechanisms by which exercise is thought to improve insulin resistance (Shulman, 2014). Additionally, the anti-inflammatory effects of exercise may be one further mechanism through which exercise is able to reduce insulin resistance and T2DM risk (Gleeson et al., 2011) and this is discussed further on in this thesis in Section 1.4.

1.3.2 High-intensity interval training.

Recently, high-intensity interval training (HIIT) has gained popularity as a time-efficient exercise option. HIIT involves repeated bursts of vigorous exercise interspersed with light activity or rest. The high intensity intervals are typically performed above 80% HR_{PEAK} and can range in duration from a few seconds to several minutes. Once a training method used primarily by athletes, HIIT has been shown to be a viable training option in clinical settings, including cardiac rehabilitation (Rognmo et al., 2012), T2DM (Little et al., 2011), and in previously inactive individuals (Child et al., 2013). These studies have provided preliminary evidence on the safety of implementing HIIT in clinical populations (Rognmo et al., 2012) and the impact of HIIT for individuals with T2DM has been recently reviewed (Francois & Little, 2015). There are numerous HIIT protocols, each involving

different interval numbers, lengths, and intensities. This heterogeneity makes comparisons between HIIT studies inherently difficult (Little et al., 2011; Little et al., 2011; McRae et al., 2012; McRae et al., 2012; Rakobowchuk et al., 2008) One practical prescription of HIIT involves one minute at 85-95 % HR_{PEAK} separated by one-minute of easy recovery, repeated 10 times, for a total work out time of 20 minutes (Little, Jung, Wright, Wright, & Manders, 2014; Little et al., 2011). This 10 x 1-min @ ~85-95 HR_{PEAK} HIIT protocol has been shown to be particularly effective at lowering blood glucose levels in individuals with T2DM (Gillen et al., 2012; Little et al., 2011) and obesity (Little et al., 2014).

Despite the lower time commitment compared to moderate intensity continuous training (MICT), HIIT has been shown to result in a number of remarkably similar physiological adaptations (Gibala, Little, MacDonald, & Hawley, 2012). For example, studies demonstrate improved muscle oxidative capacity (Little, Safdar, Wilkin, Tarnopolsky, & Gibala, 2010), and classical endurance-type training adaptions such as decreased glycogen utilization and lactate production during moderate-intensity exercise (Gibala & McGee, 2008; Rakobowchuk et al., 2008).

There are a number of speculated mechanisms through which HIIT may induce these adaptations. One mechanism may occur within skeletal muscle, where higher intensity exercise may lead to greater changes in ATP:ADP/AMP ratio (Chen et al., 2000) and activation of AMPK (Gibala et al., 2009), leading to enhanced PGC-1α activation and mitochondrial biogenesis (Egan et al., 2010). As a functional result

of these changes, the evidence to support HIIT as an effective, time-efficient alternative training option to the more traditionally prescribed MICT is accumulating.

1.3.2.1 HIIT and insulin resistance.

Even when caloric output of a HIIT session is matched to that of a MICT session, the different physiological demands placed on the body during HIIT may contribute to improvements in insulin sensitivity and glucose control that are greater than that seen after MICT. Increased muscle glycogen depletion and/or muscle fiber recruitmentmay lead to post-exercise insulin sensitivity and muscle glucose uptake that is greater in HIIT than MICT, leading to superior improvements in glycemic control (Francois et al., 2014; Little et al., 2014; Roberts, Little, & Thyfault, 2013). In addition higher intensity exercise has been shown to result in increased AMPK activation (Egan et al., 2010) and increased AMPK signaling may be the driving force for enhanced glucose uptake, glycogen synthesis and insulin signaling after exercise (Friedrichsen et al., 2013). These multiple factors may be the mechanisms underlying HIIT-induced improvements in insulin resistance and overall cardiometabolic health.

1.3.2.2 HIIT compared to MICT.

Some studies have reported superior improvements in cardiorespiratory fitness following time- and work-matched HIIT compared to MICT (Molmen-Hansen et al., 2012; Tjonna et al., 2008; Wisloff et al., 2007); however, this is not agreed upon by all (Roditis et al., 2007). Improvements in vascular endothelial function with HIIT (Moholdt et al., 2009; Tjonna et al., 2008; Wisloff et al., 2007) compared to

MICT may be attributed to the increase in shear stress, which is proportional to exercise intensity (Tinken, Thijssen, Black, Cable, & Green, 2008).

Despite the positive cardiometabolic health benefits of HIIT, it is difficult to compare studies due to the different HIIT protocols employed by various research groups. In an effort to establish the effect of HIIT on cardiorespiratory fitness, a review and meta-analysis was conducted (Weston, Wisloff, & Coombes, 2014). In ten HIIT vs. MICT matched studies on individuals with cardiometabolic disease, HIIT significantly improved VO_{2PEAK} by 19.4% compared to 10.3% with MICT (Weston et al., 2014). A larger systematic review (Kessler, Sisson, & Short, 2012) indicated that HIIT is comparable, if not superior to MICT, over a wide range of cardiometabolic health outcomes. The impact of HIIT on inflammatory parameters has not been adequately studied and neither of these systematic reviews addressed how HIIT compared to MICT for reducing inflammation. Further studies in individuals at elevated risk of T2DM are required to investigate how HIIT compares to MICT for improving glucose control and to explore the underlying mechanisms through which HIIT may impart superior cardiometabolic health benefits.

1.4 Exercise and inflammation.

Regular low- and moderate-intensity exercise have been linked to improved immune function and are known to have an anti-inflammatory effect resulting in a reduction in circulating pro-inflammatory markers (Petersen & Pedersen, 2005). This systemic anti-inflammatory effect may help explain the global benefits of exercise for reducing the risk of numerous chronic diseases. Given the aforementioned role of inflammation in promoting and sustaining insulin resistance, the ability of exercise to

reduce inflammation may be another reason, in addition to directly increasing muscle insulin sensitivity and improving cardiovascular health, for the vast benefits of exercise in individuals with prediabetes and T2DM.

1.4.1 Inflammation: acute versus chronic exercise.

There appears to be a dichotomy between the inflammatory responses to an acute bout of exercise versus chronic exercise training. Acute exercise can result in an increase in inflammation, driven primarily by leukocytosis, which is an increase in circulating immune cells (Gleeson et al., 2013). The increased mobilization of leukocytes spikes immediately post exercise and elevated levels can be measured for up 8 hours post exercise; however, these numbers return to normal resting levels within 24 hours (Robson, Blannin, Walsh, Castell, & Gleeson, 1999). Leukocytosis occurs mostly in response to demarginalization of leukocytes (Gleeson et al., 2013) as a result of increased circulating cortisol after exercise (Anane et al., 2009), with a small contribution from plasma fluid loss (Shek, Sabiston, Buguet, & Radomski, 1995). This is in contrast to regular exercise training (i.e., repeated exposure to acute bouts of exercise), where there is an overall anti-inflammatory effect (Gleeson et al., 2011).

While leukocyte numbers at rest do not appear to be different between trained and untrained individuals (Moyna et al., 1996), there is research to support decreased leukocyte numbers following training in obese or previously inactive individuals (Johannsen et al., 2012; Michishita, Shono, Inoue, Tsuruta, & Node, 2010). Long-term training intervention studies in both trained and untrained individuals have demonstrated that MICT can reduce basal expression of pro-

inflammatory cytokines (Flynn et al., 2003) and decrease LPS-stimulated production of pro-inflammatory cytokines from immune cells (McFarlin et al., 2006). Other mechanisms by which exercise training may improve immune function are outlined below.

1.4.1.1 Changes in immune cell populations with exercise.

In order to quantify the change in the inflammatory environment after exercise, many studies have measured subpopulations of leukocytes after both acute and chronic exercise. It is important to note that while many studies discuss a dose response of exercise intensity on leukocytosis, it is questionable whether these findings can be applied to HIIT due to the unique stimulus of the intermittent exercise (Weston et al., 2014).

1.4.1.1.1 Neutrophils and exercise.

As neutrophils are already the most abundant leukocyte, neutrophilia, or increased neutrophils contributes the most to leukocytosis after exercise (Gleeson et al., 2013). The neutrophilia response to exercise is fast, doubling the population in minutes in response to high-intensity exercise and increasing up to 4-fold in response to longer endurance exercise (Robson et al., 1999). A decrease in circulating neutrophils has been shown after training in overweight/obese women (Michishita et al., 2010) although other longitudinal studies have shown no effect of training on neutrophil number (Woods et al., 1999).

1.4.1.1.2 Monocytes and exercise.

Monocytes appear to increase in response to acute aerobic (Booth et al.,

2010; Simpson et al., 2009) and resistance (Simonson & Jackson, 2004) exercise. The duration of the increase in monocytes is shorter compared to neutrophils, with levels returning to the basal state within two hours of the completion of exercise (Shek et al., 1995). In response to training interventions, monocyte numbers have been shown to decrease in populations that already have elevated basal monocyte concentrations (e.g., obese, prediabetic individuals; Johannsen et al., 2012).

1.4.1.1.3 Lymphocytes and exercise.

The heterogeneous nature of lymphocytes make their response to exercise the most complicated to quantify. In general the response is biphasic, with an initial lymphocytosis phase; however, unlike neutrophils and monocytes, within 60 minutes of exercise cessation there appears to be a decrease in lymphocyte numbers to below baseline values (Shek et al., 1995), also known as lymphocytopenia. Within two days of recovery lymphocyte numbers are restored to basal values (Shek et al., 1995). There is little evidence to support changes in lymphocyte numbers with chronic training in young healthy individuals as circulating lymphocytes numbers are the same between trained and untrained (Moyna et al., 1996) and after training interventions in older adults (Simpson & Guy, 2010).

1.4.2 Anti-inflammatory mechanisms of exercise.

The number of circulating immune cells may have little association with cellular immune function (Gleeson et al., 2013) and as exercise has a limited and varied effect on cell numbers there are a number of proposed mechanisms through which exercise exerts functional changes to the immune system that result in anti-inflammatory effects. The three most recognized mechanisms are: (1) A reduction in

adipose tissue mass; (2) The development of an anti-inflammatory environment via the release of anti-inflammatory cytokines; and (3) Changes in TLR expression and activity. Regardless of the mechanisms, the acute anti-inflammatory effects of exercise could play a role in reducing chronic low-grade inflammation through an accumulative effect whereby each session of exercise throughout a training program reduces chronic low-grade inflammation and relieves insulin resistance in individuals with metabolic disease.

1.4.2.1 Reduction of adipose tissue mass.

As noted above, increased adipose tissue mass contributes to chronic low-grade inflammation primarily through infiltration of pro-inflammatory immune cells into visceral adipose tissue (VAT) (Weisberg et al., 2003). Therefore, a reduction in VAT may be instrumental in reducing inflammation. Some studies report that a large proportion of the anti-inflammatory effects of exercise can be attributed to weight loss (Fisher et al., 2011; Mediano et al., 2013). However, other studies have shown that exercise training in the absence of weight loss still results in a reduction in inflammatory markers (Dekker et al., 2007; Oberbach et al., 2006). In addition, a single bout of exercise, in which there is no appreciable change in body mass or adipose tissue, can have anti-inflammatory effects (Starkie, Ostrowski, Jauffred, Febbraio, & Pedersen, 2003). This indicates there are most likely other mechanisms through which exercise can reduce inflammation in addition to reducing VAT (Kadoglou et al., 2007; Kirwan, Solomon, Wojta, Staten, & Holloszy, 2009). Regardless, research into the mechanisms responsible for the anti-inflammatory effects of exercise needs to take into consideration changes in adipose tissue.

Training interventions that minimize the changes in VAT are important to isolate anti-inflammatory mechanisms not associated with reductions in adipose tissue.

1.4.2.2 Anti-inflammatory environment development.

During sustained aerobic exercise there is a marked increase in circulating IL-6, which is thought to be released from contracting skeletal muscle (Pedersen & Febbraio, 2008). Muscle-derived IL-6 is hypothesized to promote an anti-inflammatory cascade, including the induction of IL-10, IL-RA and cortisol (Steensberg, Fischer, Keller, Moller, & Pedersen, 2003). The increase in IL-6 and resulting anti-inflammatory effects are transient and appear to have a dose response relationship in proportion to the duration and intensity of exercise (Pedersen & Febbraio, 2008). This, in conjunction with lymphocytopenia, may explain the immunosuppression seen in elite athletes after prolonged, intense exercise (Walsh et al., 2011). However, while the development of an anti-inflammatory environment is most potent after longer duration high-intensity exercise (e.g., ultra-endurance race), the anti-inflammatory effects of exercise can also be seen after short duration, low to moderate intensity exercise (Gleeson et al., 2011) and this indicates there may be other mechanisms in addition to muscle IL-6 production that act to reduce inflammation after exercise.

1.4.2.3 Changes in TLR expression and activation.

The third potential mechanism linking exercise to reduced inflammation and the primary mechanism investigated in this thesis is a decrease in TLRs, specifically TLR2 and TLR4. TLR2 and TLR4 are markers of chronic low-grade inflammation and are increased in conditions of hyperglycemia (Dasu et al., 2008). TLR

expression on circulating monocytes is reduced after an acute bout of exercise and is lower in trained older females compared to age-matched untrained females (McFarlin, Flynn, Campbell, Stewart, & Timmerman, 2004). Furthermore, monocyte TLR2 and TLR4 expression were reduced after 12 weeks of combined resistance and MICT in previously inactive young and old participants (Stewart et al., 2005). These findings highlight reduced TLR expression as a possible direct mechanism through which exercise may diminish chronic low-grade inflammation and improve insulin resistance. Currently there is limited literature on the impact of exercise training on TLR expression on multiple immune cell types in individuals with prediabetes or T2DM. In addition, the effect of HIIT versus MICT on immune cell TLRs has not been studied.

1.5 Research overview, hypothesis and aims.

1.5.1 Summary.

There are limited studies comparing HIIT to MICT in previously inactive adults who are at an increased risk of developing T2DM. Specifically, I am unaware of any studies that compare the effects of these two exercise types on markers of inflammation, specifically TLR2 and TLR4 on immune cells. The overall purpose of this thesis was to investigate the effect of two weeks of HIIT versus MICT on cardiometabolic health and inflammatory profiles in previously inactive individuals at an elevated risk of developing T2DM.

1.5.2 Hypothesis.

The central hypothesis tested in this thesis was that HIIT would be more

effective than MICT for improving cardiorespiratory fitness, metabolic markers, and inflammatory parameters in individuals at increased risk of developing T2DM. Although MICT is clearly an effective therapeutic exercise stimulus, HIIT provides a time efficient option that has previously been shown to be more effective at improving VO_{2PEAK} in a variety of both healthy and clinical populations (Weston et al., 2014). A short-term (two-week) model of training was purposefully chosen to minimize the impact of exercise on weight/adipose loss in an attempt to investigate the direct effects of HIIT compared to MICT on improving cardiorespiratory fitness, glucose control, circulating cytokines, immune cell TLR2 and TLR4 expression and immune cell activation.

1.5.3 Aims.

1. *Does two weeks of HIIT or MICT result in an improvement in cardiorespiratory fitness as measured by VO_{2PEAK} and, if so, is there a difference between HIIT and MICT?*

One previous study has indicated a significant improvement in VO_{2PEAK} after only 7 days of HIIT (McKay, Paterson, & Kowalchuk, 2009), so I expect that two weeks of training will be sufficient time to induce significant increases in $VO_{2PEAK.}$ Studies comparing HIIT to MICT and other forms of training in both healthy and clinical populations have indicated that HIIT results in equal or superior improvements in cardiorespiratory fitness over 6 - 16 weeks of training (Gibala et al., 2006; Little et al., 2010; Rakobowchuk et al., 2008). **I hypothesized that HIIT would result in a greater improvement in cardiorespiratory fitness as measured by VO_{2PEAK}** (Hypothesis 1).

2. *Does two weeks HIIT or MICT result in an improvement in glucose control and insulin sensitivity as measured by fasting glucose, fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) and, if so, is there a difference between HIIT and MICT?*

An acute bout of exercise is known to improve insulin sensitivity and glucose control for up to 48 hours into recovery (Boule et al., 2005). The positive benefit of a single bout of exercise has been extended to short-term HIIT interventions with studies showing improvements in HOMA-IR (Hood, Little, Tarnopolsky, Myslik, & Gibala, 2011) and hyperinsulinemic-clamp derived insulin sensitivity (Richards et al., 2010) after two weeks of HIIT. **I hypothesized that HIIT would result in greater improvements in insulin sensitivity and glucose control when compared to MICT** (Hypothesis 2).

3. *Does two weeks of HIIT or MICT result in reduced inflammation as measured by basal circulating cytokines and TLR expression on immune cells and, if so, is there a difference between HIIT and MICT?*

Previously published data (Flynn et al., 2003) indicates that increased cardiorespiratory fitness is associated with a reduction in TLR expression. Increased physical activity has also been linked to a reduction in circulating pro-inflammatory cytokines (Gleeson et al., 2011). As I expected HIIT would result in greater improvements in cardiorespiratory fitness, glucose control, and insulin sensitivity, **I hypothesized that HIIT would result in greater reductions in**

circulating pro-inflammatory cytokines and TLR expression (Hypothesis 3).

4. *Does two weeks of HIIT or MICT alter immune cell function as measured by ex vivo LPS- and PAM-CSK4-stimulated whole blood cultures and, if so, is there a difference between HIIT and MICT?*

LPS is a TLR4 ligand that promotes pro-inflammatory cytokine production from immune cells, particularly CD14+ monocytes (Kaisho & Akira, 2006). PAM-CSK4 acts to similarly increase pro-inflammatory cytokine secretion from immune cells via TLR2 agonism. Cytokine production as measured via *ex vivo* whole blood culture stimulation with LPS and PAM-CSK4 gives a measure of the responsiveness of circulating blood cells to an inflammatory trigger. Previous research has indicated that exercise training reduces LPS-induced cytokine production for some cytokines (e.g., IL-6), while not impacting others (e.g., TNF-α; Stewart et al., 2005). With the hypothesized larger increase in fitness and greater reduction in TLR expression with HIIT, **I hypothesized that HIIT would result in a larger decrease in LPS- and PAM-CSK4-stimulated cytokine release compared to MICT** (Hypothesis 4).

2.0 Manuscript from thesis data

The following modified manuscript titled “Short-term high-intensity interval and moderate-intensity continuous training reduce leukocyte TLR4 in inactive adults at elevated risk of type 2 diabetes” was written based on the results of this thesis project. It was accepted by the Journal of Applied Physiology on June 30, 2015 and published online ahead of print on July 2, 2015 (Available at: http://jap.physiology.org/content/early/2015/07/02/japplphysiol.00334.2015) .

2.1 Introduction.

Chronic low-grade inflammation, characterized by an increase in basal circulating pro-inflammatory cytokines and/or acute phase reactants (Dandona et al., 2004) is implicated in the pathogenesis of obesity, insulin resistance, and T2DM (Lumeng & Saltiel, 2011). While the underlying cause of inflammation has not yet been fully elucidated, metabolic disruptions associated with insulin resistance have been shown to directly trigger innate immune responses. For example, hyperglycemia and an elevation in free fatty acids are linked with increased activation of immune cells, including elevation in surface protein expression of TLRs (Dasu et al., 2008) and augmented release of pro-inflammatory cytokines (Morohoshi et al., 1996). Studies have also reported elevated CD14+ monocyte TLRs in patients with T2DM compared to age-matched normoglycemic controls (Dasu et al., 2010). TLRs are conserved pattern-recognition receptors that recognize a variety of exogenous and endogenous pathogens to coordinate innate immune responses (Akira & Hemmi, 2003). Increased TLR2 and 4 expression and the resulting pro-inflammatory environment are associated with a cluster of

cardiometabolic risk factors, including insulin resistance, T2DM and atherosclerosis (Dasu et al., 2010).

Exercise improves metabolic health and decreases the risk of T2DM in individuals with prediabetes (Colberg et al., 2010). One potent systemic benefit of regular exercise is thought to be its anti-inflammatory effects (Petersen & Pedersen, 2005). Some of the anti-inflammatory effects of regular exercise are likely attributable to a reduction in adipose tissue (Fisher et al., 2011) but there is also growing evidence that exercise, in the absence of weight loss, can directly impact immune cell phenotype and alter systemic inflammatory mediators (For review see (Gleeson et al., 2011). The ability of exercise to reduce monocyte TLRs is one hypothesized mechanism through which this anti-inflammatory effect may occur (Stewart et al., 2005). Multiple studies have shown reduced monocyte TLR4 expression after both acute exercise and training interventions (Flynn et al., 2003; Stewart et al., 2005) but the impact of exercise on monocyte TLR2 is less clear (Simpson et al., 2009) The influence of aerobic exercise on TLR expression on other distinct immune cells has not been adequately studied. One study examining TLR2 and TLR4 in mixed peripheral blood mononuclear cells (PBMCs) reported no effects after 15 days of aerobic exercise training (Reyna et al., 2013). As PBMCs represent mostly lymphocytes with some monocytes, TLR expression on isolated cell types cannot be quantified using this technique.

Despite evidence for the anti-inflammatory impact of exercise, it is currently unclear what type or intensity of exercise is most effective (Gleeson et al., 2011). HIIT has gained recent attention as a time-efficient exercise strategy, providing a

unique physiological stimulus compared to traditionally prescribed MICT (Weston et al., 2014). A recent meta-analysis of ten studies reported greater improvements in cardiorespiratory fitness following HIIT (average increase of 19.4%) compared to MICT (average increase of 10.3%) in patients with cardiometabolic disease (Weston et al., 2014). Studies have also reported greater improvements in endothelial function (Tjonna et al., 2008) and glucose control (Little et al., 2014) after HIIT compared to traditional MICT in overweight/obese individuals. Based on the findings that HIIT promotes superior gains in cardiometabolic health, it is possible that HIIT may also have greater anti-inflammatory effects compared to MICT, but this hypothesis has not been tested.

The purpose of this study was to examine the impact of HIIT and MICT on markers of inflammation and cardiometabolic health in individuals at elevated risk of T2DM. We examined: 1) circulating pro- and anti-inflammatory cytokines; 2) leukocyte TLR2 and 4 expression; 3) *ex vivo* cytokine secretion in whole blood cultures; and 4) standard cardiometabolic health markers, before and after two weeks of HIIT or MICT in inactive, overweight/obese adults. We employed short-term training modeled after previous research (Little et al., 2011) to minimize any changes in body composition in order to isolate the direct effects of HIIT and MICT on inflammatory parameters. We hypothesized that HIIT would result in greater improvements in cardiometabolic health when compared to MICT. Due to the links between improved cardiorespiratory fitness (Flynn et al., 2003; McFarlin et al., 2006) and metabolic health (Dasu et al., 2010) with reduced inflammation, we also tested

the hypothesis that HIIT would lead to greater reductions in markers of inflammation when compared to MICT.

2.2 Methods and Materials

2.2.1 Participants

Participants recruited were considered to have prediabetes based on HbA1c values between 5.7 – 6.4% (American Diabetes Association, 2013 Bayer A1C Now, United States), and/or a Canadian Diabetes Risk assessment questionnaire score above 21 (CanRISK, Public Health Agency of Canada., 2011). Additionally in order to be eligible participants had to be inactive (assessed by standard 7-day physical activity recall interview conducted during screening and defined as completing less than two 30 minute bouts of moderate-to-vigorous physical activity per week) and cleared for participation in vigorous activity as determined by the Canadian Society for Exercise Physiology (CSEP) Physical Activity Readiness Questionnaire-Plus (PAR-Q+) administered by a CSEP Certified Exercise Physiologist®. Exclusion criteria included diagnosed diabetes, glucose lowering medications, uncontrolled hypertension (blood pressure >160/90 mmHg), history of heart disease, myocardial infarction or stroke, and any other contraindications to exercise. All subjects provided written informed consent. The study was approved by the University of British Columbia Clinical Research Ethics Board. A total of 62 participants were screened and 39 were eligible for the study and enrolled. Demographics and baseline characteristics are provided in Table 1.

Table 1: Baseline characteristics of the participants before starting the two-week training.

	HIIT (20)	MICT (18)
% Female	85 %	78 %
Age (years)	52 (10)	52 (10)
Canrisk score	29 (10)	33 (11)
HbA1c (%)	6.0 (0.5)	5.5 (0.4)
Systolic blood pressure (mmHg)	133 (17)	131 (8)
Diastolic blood pressure (mmHg)	83 (7)	82 (8)
VO_2peak (ml/kg/min)	20.4 (3.4)	20.3 (5.0)
Body mass index (BMI; kg/m^2)	32.9 (6.6)	31.4 (4.1)
Anti-depressants (n)	5	7
Anti-hypertensive (n)	1	3
Thyroid medication (n)	1	2

Values are means (SD). Independent t-tests showed no differences between the two randomized groups at baseline (all P>0.05).

2.2.2 Experimental Protocol.

After screening eligible participants were randomized into HIIT (n=20, 3 males, 17 females) or MICT (n=19, 4 males, 15 females) groups. Both groups completed the same experimental protocol, which consisted of baseline (pre) testing, a ten session exercise training intervention over a two week period, and post-testing conducted 48 - 72 hours following the final training bout in order to avoid confounding influence from the last training bout (Durrer et al., 2015).

2.2.2.1 Pre-Testing.

Pre-testing was conducted at least seven days prior to the training program start date when participants had no current or recent infection symptoms (assessed through self report). The morning after an overnight (>8 h) fast, manual blood

pressure (BP) was measured using Canadian Hypertension Education Program guidelines (Dasgupta et al., 2014) and a blood sample was obtained from an antecubital vein by venipuncture. Body mass and height were assessed. Participants consumed a light snack prior to completing a continuous incremental ramp maximal exercise test on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine VO_{2PEAK}, maximal heart rate (HR_{PEAK}), and peak power output (Wpeak). The test started at 50 Watts and increased by 15 Watts/min until volitional exhaustion and/or revolutions per minute fell below 50. Continual measures of oxygen uptake (VO_2) and carbon dioxide output (VCO_2) were made by a metabolic cart (Parvomedics TrueOne 2400, Salt Lake City, Utah, USA). The metabolic cart was calibrated with standard medical-grade gases and a 3.0 L syringe before every test. VO_2peak was defined as the highest 30-sec average for VO_2 (L/min and ml/kg/min). HRmax and Wpeak were defined as the highest value achieved.

2.2.2.2 Training intervention.

The training program was comprised of ten progressive sessions of exercise performed over a two-week period. Exercise sessions for HIIT and MICT were designed to be matched for external work based on calculations of %Wpeak obtained on the VO_{2PEAK} test. Specifically, individuals randomized to HIIT began with four intervals lasting 1-minute each at ~85-90% Wpeak (eliciting ~85-90% HRpeak) and increased to 10 X 1-min intervals by day 10. The interval protocol had a work:rest ratio of 1:1, with 1-min recovery intervals completed at 20% of Wpeak and included a 3 minute warm-up and cool-down at 32.5% Wpeak. This HIIT protocol

was modeled after previous studies that indicate improvements in cardiometabolic health in individuals with, and at risk for, T2DM (Little et al., 2014; Little et al., 2011). Individuals randomized to MICT began with 24 minutes of continuous activity at ~32.5% Wpeak (eliciting ~60-65% HRpeak; Little et al., 2014) and gradually increased at the same percentage increase in estimated total work up to a duration of 50 minutes by day 10 (Table 2). Estimated external work output for each session is described in Table 5 (*appendix 1.9*).

Table 2. Daily exercise prescription for HIIT and MICT over the two-week intervention.

HIIT (85 – 90% of HRpeak)	Training day	MICT (60 - 65% HRpeak)
4 intervals	1	24 minutes
5 intervals	2	29 minutes
6 intervals	3	33 minutes
6 intervals independently.	4	33 minutes independently
7 intervals	5	36 minutes
8 intervals	6	40 minutes
8 intervals independently.	7	40 minutes independently
9 intervals	8	43 minutes
9 intervals independently.	9	43 minutes independently
10 intervals	10	50 minutes

Participants completed the first and last session on a cycle ergometer at these prescribed intensities but were allowed to choose between treadmill walking, outdoor walking, elliptical trainer, or cycle ergometer for other sessions. During the intervention participants wore downloadable Polar heart-rate monitors (Polar FT7, Kempele, Finland) to ensure that training elicited the prescribed heart rate. Two trained research assistants supervised participants for seven of the 10 exercise bouts in the lab. Three bouts (one in week one and two in week two) were performed independently as outdoor walking by participants at their home, using heart rate monitors to ensure compliance.

2.2.2.3 Post-testing.

Approximately 48-72 hours after the last training session participants returned to the lab for post-testing, which was conducted in an identical manner as baseline testing.

2.2.3 Blood Measures.

2.2.3.1 Metabolic markers.

Venous blood samples were obtained by venipuncture using a 21 g needle (BD Eclipse) into sodium heparin tubes (BD Vacutainer). A portion of the sample was centrifuged at 1200g to obtain plasma and used for analyses. Fasting glucose was measured by the hexokinase method (*details appendix 1.1*), fasting NEFA assessed by colorimetric assay (Wako Chemicals, Texas, USA, *details in appendix 1.2*), and fasting insulin was measured by ELISA (Mercodia, Upsulla, Sweden, *details in appendix 1.3*) according to manufacturer's instructors using on a clinical chemistry analyzer (Chemwell 2910, Awareness Technologies), all in duplicate with an average coefficient of variation (CV) <4% between duplicates. HOMA-IR and beta cell function (HOMA-β) was calculated using the calculator provided by University of Oxford, Diabetes Trial Unit (www.dtu.ox.ac.uk/homacalculator, *further details in appendix 1.4*). Plasma fructosamine was assessed by automated commercial assay (DZ-112B-K, Diazyme, Poway, California, USA) using the aforementioned clinical chemistry analyzer. The CV of duplicates was <10%.

2.2.3.2 TLRs.

Whole blood was analysed using flow cytometry to determine TLR2 and TLR4 expression on CD14+ monocytes, CD15+ neutrophils, and lymphocytes. Blood was kept on ice and analyzed within 45 minutes of collection. 6 µl of FcR blocking reagent was added to 54 µl of whole blood and incubated in the dark for 10 min at 4°C. Blood was stained with 10 µl of each of CD45-APC-Vio770, CD14-VioBlue, CD15-FITC, TLR2-PE, and TLR4-APC (all from Miltenyi Biotech, Bergisch Gladbach, Germany) resulting in a 1:11 dilution of all antibodies. Samples were again incubated at 4 °C in the dark for 10 min. 1 ml of MACSQUANT® running buffer (Miltenyi Biotech, Bergisch Gladbach, Germany) was added to each of the samples, which were subsequently analyzed on a Miltenyi MACSQuant Analyzer 10 flow cytometer using a no-wash, no-lyse protocol. The trigger was set on the CD45+ channel to capture leukocyte events while red blood cells and debris were omitted based on lack of CD45 expression and scatter profile. Monocyte, neutrophil, and lymphocyte gates were established based on characteristic forward and side scatter as well as staining positive for CD14 (monocytes) and CD15 (neutrophils). Expression of TLR2 and TLR4 on CD14+ monocytes, CD15+ neutrophils, and lymphocytes were assessed as median fluorescence intensity (MFI) using MACSQuant Software. Fluorescence minus one controls (FMO) were used to identify positive TLR2 and TLR4 events. The gating strategy is shown in Figure 4 (*further details of the protocol are in appendix 1.5*).

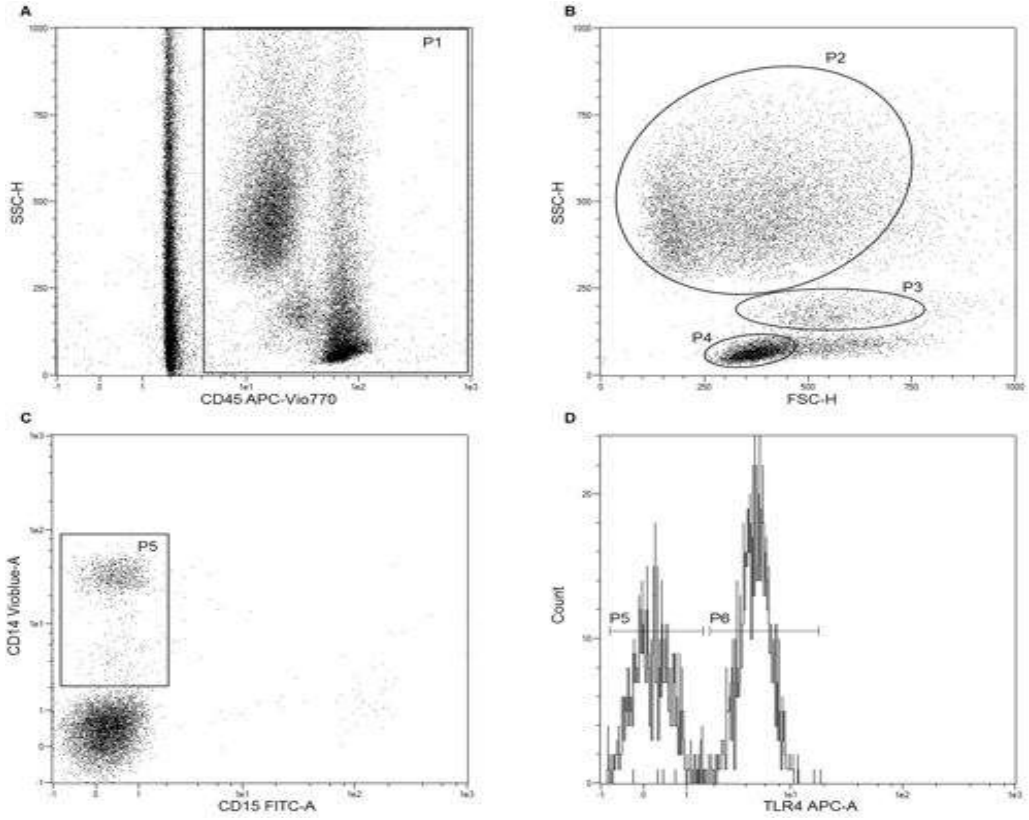


Figure 4: Flow cytometry gating strategy used to quantify toll-like receptors (TLRs) on immune cell subtypes Example flow cytometry plots of (**A**) Gating cells with CD45+ trigger (P1), (**B**) Identifying monocyte (P3), lymphocyte (P4) and neutrophil (P2) populations based on scatter (**C**) Gate for CD45+/CD14+ monocytes (P5), and (**D**) Histogram of TLR4 expression on CD14+ monocytes (P6) with corresponding fluorescence minus one (FMO, P5) control shown.

2.2.3.3 Whole blood cultures.

Whole blood cultures were prepared by diluting blood 10 times in serum-free RPMI media (Sigma) supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml) containing 5 mM glucose and seeding cells in 24-well cell culture plates at 540 µl per well as we have described previously (Wan, Durrer, Mah, Simtchouk, & Little, 2014). Cultures were stimulated with i) the TLR4 agonist bacterial lipopolysaccharide (LPS, from *Escherichia coli* 055:B5; L6529, Sigma) at 100 pg/ml; and ii) the synthetic bacterial lipoprotein TLR2 agonist PamCSK4 (L2000, EMC

microcollections, Tuebingen, Germany) at 100 ng/ml. Unstimulated cultures served as a control to confirm cytokine induction with LPS and PamCSK4. After 24 h of incubation at 37 °C in 5% CO_2, blood culture plates were centrifuged at 2000 g for 15 min at 4 °C (*appendix 1.6*) and supernatants were analyzed for tumor necrosis factor – α (TNF-α), Interleukin (IL) -1β, IL-6 and IL-10 using ELISA (R and D Systems, DuoSet human cytokine ELISA, *see appendix 1.7*) according to the manufacturer's instructions. Absorbance was read at 450 nm using a FluoStar Omega plate reader (BMG Labtech, Ortenburg, Germany) and cytokine secretion was expressed per CD45+ leukocyte as determined by flow cytometry.

2.2.3.4 Plasma cytokines.

Fasting plasma concentrations of TNF-α, IL-1β, IL-6 and IL-10 were analyzed by multiplex immunoassay (Custom MILLIPLEX panel, Millipore, Massachusetts, USA) and read on a MAGPIX™ Bio-Plex® reader (BioRad, Hercules, CA, *appendix 1.8*) according to the kit manufacturer's instructions. Plasma was centrifuged at 10,000 g for 10 min at 4°C to remove any debris and was analyzed in duplicate. The average CV for duplicates was <6%. Results were analyzed using Bio-Plex® Manager 6.1 Software.

2.2.4 Statistical Analysis.

Data were analyzed using SPSS Statistics (v22, 2013). Normality was assessed using Q-Q plots and the Shapiro-Wilk test. Non-normal data was log transformed for analyses. Comparisons of baseline values between the two groups were analyzed with unpaired t-test. A series of two-factor (group X time) repeated measures ANOVA were used to examine changes in cardiometabolic variables,

anthropometrics and markers of inflammation. Fisher LSD post-hoc tests were used to probe significant interactions. Statistical significance was set at P≤0.05. Effect sizes were calculated using Cohen's d.

2.3 Results.

Of the 39 participants who were eligible and enrolled in the study, 20 were randomized to HIIT and 19 randomized to MICT. All completed the two-week training period with no adverse effects, however one female participant in MICT did not complete post-testing (no reason provided, did not respond to phone calls). This participant was removed from analyses resulting in final data available for 20 participants in HIIT and 18 in MICT. There were no significant differences between the two groups at baseline (see Table 1). Expected workload intensity during training was confirmed with the average heart rate elicited during HIIT being 82 ± 6 % and MICT being 69 ± 7 % (calculation included warm up and cool down). There was no difference between average %HRpeak during supervised sessions and home sessions (HIIT 82 ± 4 % vs. 82 ± 4 % and MICT 69 ± 6 % vs 69 ± 4 %).

2.3.1 Anthropometrics.

There was a significant effect of time on body mass (p=0.029, Cohen's *d*=0.11) and body mass index (BMI) (p=0.025, Cohen's *d*=0.08) with no difference between groups (group x time interaction, p's> 0.53). Body mass reduced on average by 0.54% (88.7 ± 20.1 vs. 88.0 ± 20.4 kg in HIIT, 88.0 ± 20.4 vs. 86.6 ± 13.5 kg in MICT). BMI decreased from 32.9 ± 6.6 to 32.6 ± 6.7 kg/m^2 in HIIT and 31.4 ± 4.1 to 31.3 ± 4.0 kg/m^2 in MICT. There was a significant effect of time on mean

arterial blood pressure (100 ± 9 vs. 98 ± 10 mmHg in HIIT, 98 ± 8 vs. 96 ± 7 mmHg in MICT, p=0.010, Cohen's *d*=0.22) but no differences between the groups.

2.3.2 Cardiorespiratory fitness.

Changes in cardiorespiratory fitness over the two-week intervention are shown in Figure 5. There was a significant main effect of time for absolute VO_{2PEAK} (Figure 5), relative VO_{2PEAK} (20.4 ± 3.4 vs. 21.9 ± 4.0 ml/kg/min in HIIT, 20.6 ± 4.9 vs. 22.1 ± 4.7 ml/kg/min in MICT, p<0.001, Cohen's *d*=0.35) and peak power output (152 ± 26 vs. 160 ± 29 W in HIIT, 153 ± 38 vs. 162 ± 36 W in MICT, p<.001, Cohen's *d*=0.25), indicating that both HIIT and MICT improved fitness with no difference between the two exercise conditions.

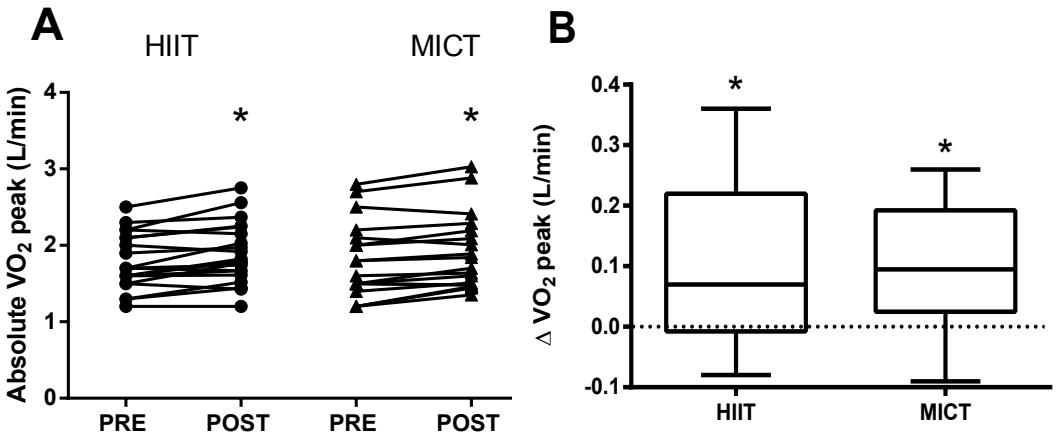


Figure 5: Two weeks of both HIIT and MICT increases cardiorespiratory fitness. Individual changes are shown by lines in (**A**) and change from pre-training is shown in the boxplot in (**B**). Main effect of time was significant (P<0.05). *significant increase from Pre-training within group (P<0.05). VO_2peak data was analyzed for n=20 in HIIT and n=18 in MICT.

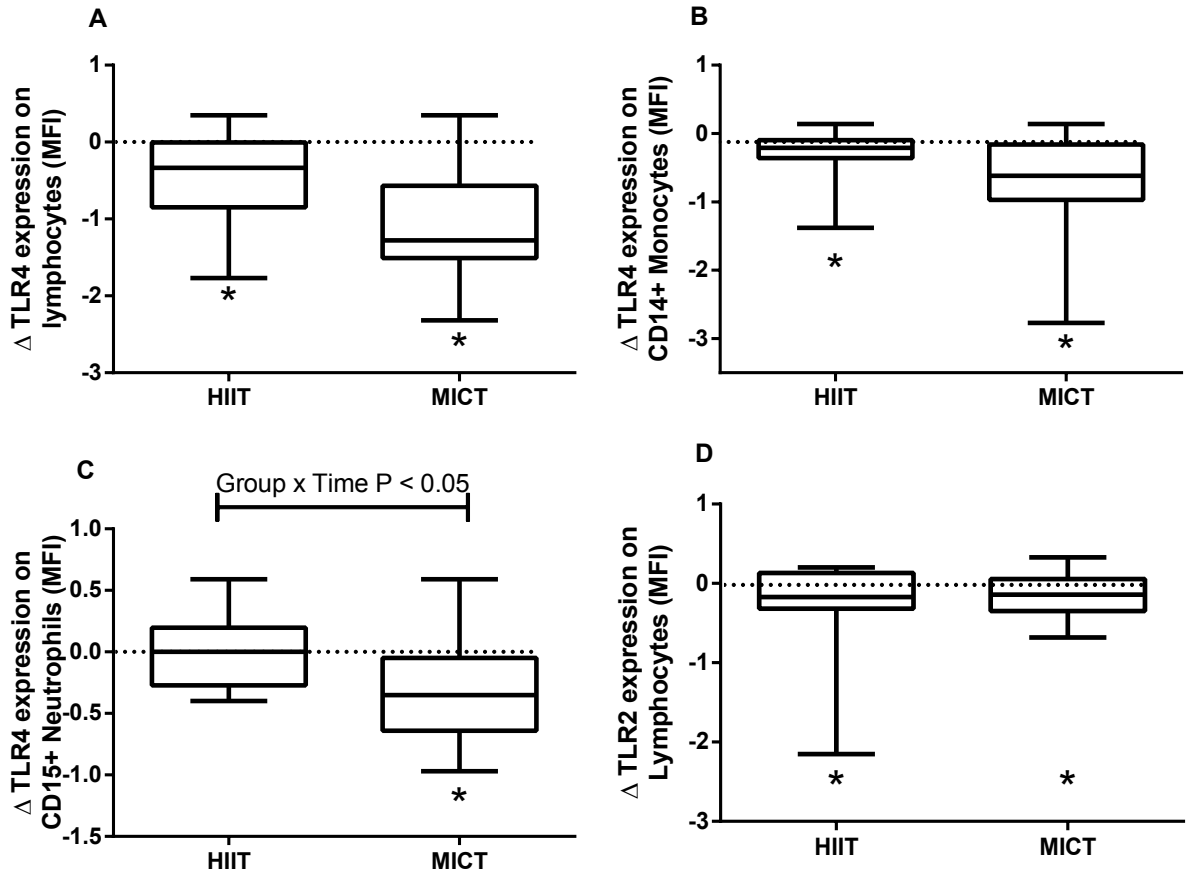


Figure 6. Two weeks of both HIIT and MICT leads to reductions in immune cell TLR expression. TLR4 measured on lymphocytes (A), CD14+ monocytes (B), CD15+ neutrophils (C) and TLR2 measured on lymphocytes (D) by flow cytometry before and after training. Main effects of time were significant for all (P<0.05) with a significant group X time interaction seen for CD15+ neutrophils *Significant difference from Pre-training within group. There were no significant changes in TLR2 on CD14+ monocytes or CD15+ neutrophils (*appendix 2.5 and 2.6*). Flow cytometry data was analyzed for n=10 in HIIT and n=15 in MICT.

2.3.3 Inflammatory markers.

TLR4 expression lymphocytes (Figure 6A) and CD14+ monocytes (Figure 6B) was reduced following training in both HIIT and MICT (main effects of time, both p<0.05, Cohen's *d*= 1.54 and 0.68 respectively, reduced by ~25% and ~15% respectively) with no difference between groups.

There was a significant group X time interaction ($p<0.018$) for TLR4 expression on CD15+ neutrophils (Figure 6C), with post-hoc tests revealing a reduction (~15%) following MICT only ($p=0.003$, Cohen's *d*=1.10), with no change in HIIT. There was a significant main effect of time for lymphocyte TLR2 ($p=0.010$, Cohen's *d*=0.54), indicating a reduction with training (Figure 6D, mean reduction of ~5%). TLR2 on monocytes and neutrophils was not affected by training (both $p>0.20$, *appendix 2.5*).

There were no significant changes in plasma cytokines (Table 3) except for an interaction effect on IL-10, with post-hoc test showing a reduction after MICT ($p<0.05$, Cohen's *d*=0.11). The LPS and PamCSK4 stimulation both led to significant induction of cytokines. As expected, cytokines were undetectable in unstimulated control cultures. There were no effects of training on cytokine induction with LPS or PamCSK4 stimulation of whole blood cultures (Table 3). Training had no effect on monocyte, neutrophil, or lymphocyte numbers (time main effects all $p>0.20$, Table 3).

Table 3: Fasting plasma cytokines (HIIT n = 17, MICT n = 14), LPS- (HIIT n = 7, MICT n = 8) and PamCSK4- (HIIT n = 8, MICT n = 8) induced cytokine release from whole blood cultures and immune cell number before and after two weeks of HIIT or MICT.

	HIIT		MICT		P-value		
Variable	Pre	Post	Pre	Post	Time	Group	Interaction
Fasting plasma cytokines (pg/ml)							
TNF-α	12.1 (3.4)	13.3 (4.0)	15.5 (5.2)	15.1 (5.4)	0.26	0.11	0.06
IL-6	2.6 (1.4)	2.8 (1.9)	2.3 (1.5)	2.0 (1.3)	0.76	0.32	0.23
IL-1β	1.3 (1.6)	1.2 (1.3)	1.0 (1.6)	1.0 (1.6)	0.99	0.54	0.60
IL-10	2.4 (2.9)	2.3 (1.8)	7.7(11.6)	6.4(11.5)	0.16	0.05	0.05
LPS-induced cytokines (pg/CD45$^+$ leuckocyte x 10^4)							
TNF-α	3.3 (3.4)	4.2 (4.2)	5.1 (5.3)	7.5 (5.8)	0.13	0.26	0.47
IL-6	8.7 (5.9)	8.3 (5.5)	7.0 (7.3)	10.0 (5.9)	0.40	0.99	0.26
IL-1β	1.3 (1.2)	1.5 (1.6)	1.1 (1.4)	2.2 (1.0)	0.22	0.66	0.40
IL-10	1.1 (0.4)	1.0 (0.3)	0.9 (0.3)	1.2 (0.4)	0.18	0.84	0.10
PamCSK4-induced cytokines (pg/CD45$^+$ leuckocyte x 10^4)							
TNF-α	2.3 (2.9)	2.4 (1.5)	2.0 (1.2)	1.4 (1.0)	0.74	0.43	0.52
IL-6	8.4 (5.2)	9.6 (4.8)	8.7 (8.1)	6.7 (7.6)	0.66	0.70	0.06
IL-1β	0.2 (0.1)	0.2 (0.2)	0.3 (0.3)	0.8 (1.4)	0.23	0.25	0.36
IL-10	1.3 (0.8)	1.1 (0.2)	1.4 (0.6)	1.1 (0.6)	0.17	0.92	0.88
Percentage of lymphocytes, CD14 monocytes, and CD15+ neutrophils relative to total live CD45+ cells							
Lymphocytes	20 (9)	19 (6)	20 (6)	21 (7)	0.96	0.68	0.30
CD14+ monocytes	6 (2)	7 (2)	6 (2)	6 (2)	0.14	0.29	0.98
CD15+	73 (10)	74 (5)	74 (7)	73 (8)	0.81	0.88	0.36

2.4.4 Metabolic measures.

Plasma fructosamine was reduced after training (main effect of time, $p<0.05$, Cohen's *d=0.40,* Table 4) with no difference between HIIT and MICT. There was a significant group X time interaction ($p=0.05$, Table 4) for fasting plasma glucose, with

post-hoc tests revealing a reduction following MICT (p=0.031, Cohen's *d*=0.30) but no change in HIIT (p=0.489). Neither HIIT nor MICT impacted fasting insulin, HOMA-IR, HOMA-β or NEFA concentrations (Table 4).

Table 4. Fasting plasma metabolic markers before and after two weeks of HIIT (n = 15) or MICT (n = 15).

		HIIT		MICT		P-value	
Variable	Pre	Post	Pre	Post	Time	Group	Interaction
Glucose (mmol/L)	5.6 (1.2)	5.7 (1.1)	5.9 (1.0)	5.6* (1.0)	0.34	0.78	0.05
Fructosamine (µmol/L)	228 (93)	177 (91)	186 (97)	161 (97)	0.02	0.37	0.40
Insulin (mU/L)	15.7 (11.4)	15.1 (11.6)	12.2 (3.5)	13.0 (5.5)	0.89	0.99	0.58
HOMA-IR	2.0 (1.4)	2.0 (1.5)	1.6 (0.5)	1.7 (0.7)	0.79	0.96	0.75
HOMA-β	112 (64)	106 (58)	94 (19)	109 (31)	0.37	0.69	0.07
NEFA (mmol/L)	0.47 (0.24)	0.49 (0.20)	0.48 (0.16)	0.48 (0.20)	0.11	0.93	0.68

Values are means (SD) *P<0.05 vs. Pre within condition

2.4 Discussion.

This study shows that, in previously inactive adults at an elevated risk of developing T2DM, two weeks of either HIIT or MICT significantly improved VO_{2PEAK} and reduced TLR4 expression on monocytes and TLR2 and TLR4 expression on lymphocytes, with no differences between exercise conditions. MICT did appear superior to HIIT for reducing fasting plasma glucose and TLR4 expression on neutrophils. Both types of training improved glucose control assessed by plasma fructosamine. A small but significant reduction in body mass and BMI was found but there were no significant changes in other markers of cardiometabolic health, including fasting plasma insulin, NEFA or HOMA-IR.

2.4.1 Changes in TLR expression.

It is currently well accepted that exercise exerts an anti-inflammatory effect (Gleeson et al., 2011) and the significant effects of short-term exercise training on TLR2/4 described in this thesis provides further evidence for this. Although previous studies have reported reduced monocyte TLR4 following exercise training (Flynn et al., 2003; McFarlin et al., 2006; Stewart et al., 2005) there has been one study reporting increased monocyte TLR4 after two weeks of HIIT (Child et al., 2013). However this is the first study, to our knowledge, to demonstrate reductions in TLR4 on neutrophils and TLR2 and 4 on lymphocytes. Both TLR2 and 4 have been implicated in the development of cardiometabolic disease and both have been reported to be elevated on monocytes in conditions of high glucose or nutrient excess (Dasu et al., 2008; Dasu et al., 2010). Agonism of both induces the release of cytokine to initiate an innate immune response. TLR2 responds primarily to gram positive bacteria and bacterial lipoproteins while TLR4 is the classic receptor for gram negative cell wall components (i.e., lipopolysaccharide; Akira & Hemmi, 2003)). However, there are many reported endogenous ligands for TLR2 and TLR4 that could perpetuate an inflammatory response in the context of obesity or metabolic disease (Akira & Hemmi, 2003; Dasu et al., 2008).

A large component of the innate immune response may have been missed by previous research, in which only changes in monocyte TLR expression are measured, as monocytes only make up 5 – 15% of circulating immune cells (Rogacev et al., 2010). A previous study also employing short-term training showed no effect on TLRs measure in isolated PBMCs (containing lymphocytes and

monocytes), however discrepancy may be explained by differences in training volume and technique of TLR measurement (western blot in PBMCs) compared to our study (Reyna et al., 2013). The reduction in TLRs on multiple immune cell types highlights a direct and systemic effect that could partly explain the anti-inflammatory response to aerobic exercise. The relative importance of differences in TLR2 and TLR4 expression across different immune cells following exercise is not known. However, based on our novel findings of changes in TLR2 and TLR4 on lymphocytes after both HIIT and MICT and TLR4 on neutrophils after MICT, it would seem that future research should examine lymphocytes and neutrophils in addition to monocytes when assessing the cellular mechanisms underlying the anti-inflammatory effects of exercise.

Overall both HIIT and MICT reduced cellular markers of inflammation but MICT led to a greater reduction in TLR4 on CD15+ neutrophils. Neutrophil TLR4 is important in the innate immune response (Hayashi, Means, & Luster, 2003) and appears to regulate neutrophil survival and activation (Sabroe et al., 2003). The reduction in neutrophil TLR4 after MICT supports lower reduced innate immune activation in this cell type and possible reductions in inflammation following training in MICT. The importance of this altered neutrophil TLR4 following exercise requires further investigation. HIIT and MICT were not matched for exercise duration, in order to maintain the time-efficiency of HIIT in this study. As a result individuals randomized to MICT spent approximately twice as much time exercising. It is possible that the longer duration of exercise and/or the moderate-intensity had greater impact on neutrophil TLR4.

2.4.1.1 Mechanisms underlying the reduction in immune cell TLR.

The mechanisms behind exercise-induced TLR4 reductions are not fully understood. Reduced expression of TLR4 may occur as a result of low-dose exposure to exogenous ligands including LPS, peptidoglycan and double stranded RNA as well as heat shock protein (HSP) (Flynn & McFarlin, 2006), all of which may increase during each exercise bout throughout a training program. Exposure to these ligands may induce TLR tolerance, which can be measured as a decrease in expression (Flynn & McFarlin, 2006). Additionally recent research indicates that acute exercise-induced changes in anti-inflammatory gene expression may have the potential to modulate TLR expression and function (Abbasi et al., 2014). It is currently unclear how potential changes in these processes culminate or interact to alter TLR expression, highlighting the need for future mechanistic research, particularly with regard to individual sessions of HIIT or MICT within a training program.

As high glucose *in vitro* and hyperglycemia *in vivo* (Dasu et al., 2008; Dasu et al., 2010) have been linked to elevated TLR4 surface expression on immune cells, exercise-mediated reductions in plasma glucose may be a potential mechanism leading to lower TLR4 surface protein expression. The reduction in plasma fructosamine, which reflects average blood glucose concentration over an ~2 week period (Goldstein et al., 2004), provides some support that improved glucose control may have contributed to lower TLRs. However, given that we found reduced fasting glucose after MICT only but reductions in TLR4 after both HIIT and MICT, reduced hyperglycemia does not seem to fully explain our findings.

Previous studies have indicated the anti-inflammatory effects of exercise are primarily mediated by weight loss (Gleeson et al., 2011; Yudkin, 2007). Using short-term training, we aimed to minimize any reductions in fat mass in order to assess the direct effects of exercise on markers of inflammation. However, there was a significant main effect of time for body mass, BMI and waist circumference, indicating that training did lead to a small, but statistically significant, reduction. Given that these reductions were minimal and had small effect sizes, we believe that reduction in immune cell TLRs were not related to changes in body mass.

2.4.2 Impact of HIIT on inflammation.

To our knowledge this is the first short-term training study to compare the inflammatory profile after HIIT versus MICT in previously inactive overweight or obese individuals at elevated risk for T2D. Our findings indicate that two weeks of HIIT has an apparent anti-inflammatory effect as measured by a reduction in monocyte TLR4 and lymphocyte TLR2 and TLR4 expression. However, our hypothesis that HIIT would lead to greater reductions in inflammatory markers was not supported. If anything, there were greater anti-inflammatory effects of MICT as supported by larger reductions in neutrophil TLR4. These findings provide preliminary evidence that moderate-intensity exercise may lead to greater anti-inflammatory responses in inactive individuals who are overweight and/or obese.

2.4.2.1 Circulating cytokines and metabolic markers.

The significant reduction in immune cell TLR2 and TLR4 occurred without a change in basal circulating pro-inflammatory cytokines, in agreement with previous research (McFarlin et al., 2004). Plasma cytokines originate from spill over from

various organs and tissues such as adipose, skeletal muscle, liver, circulating immune cells (Womack et al., 2007) and blood vessels (Lumeng & Saltiel, 2011). Thus, despite potential anti-inflammatory effects detected at the cellular level, changes in inflammatory cytokines may not be detected in plasma. We feel this highlights the additional insight provided by measuring the impact of exercise at the level of immune cells as opposed to solely assessing plasma cytokines.

Plasma IL-10, known to be anti-inflammatory (Joyce et al., 1994) was decreased with MICT only. This finding was somewhat unexpected and may warrant further study. However, it is important to recognize that an increase in plasma IL-10 may be an early compensatory response to chronic low-grade inflammation (Avdiushko, Hongo, Lake-Bullock, Kaplan, & Cohen, 2001) and thus it could be speculated that reduced plasma IL-10 is indicative of lower, as opposed to greater, inflammation. Additionally each exercise bout may result in a temporary increase in circulating IL-10 (Pedersen & Febbraio, 2008) resulting in the development of an anti-inflammatory environment after each training session.

The reduction of plasma fructosamine after both HIIT and MICT provides evidence that, over the course of the 2-week training period, overall exposure to hyperglycemia was lower. Previous research has shown reduced hyperglycemia assessed by continuous glucose monitoring following an acute session of HIIT (Little et al., 2014) and MICT (van Dijk et al., 2012) in individuals at elevated risk of T2D. Therefore, the reduction in fructosamine likely reflects the cumulative effect of glucose lowering following each bout of exercise throughout training. Despite equal effects on fructosamine, fasting plasma glucose was only reduced following MICT.

This supports previous findings that indicate duration of exercise is the most important factor for improving glucose regulation in response to exercise (Houmard et al., 2004; Newsom, Everett, Hinko, & Horowitz, 2013). One explanation for this effect of exercise duration may be that when a longer duration of exercise is prescribed, there are fewer hours in which sedentary behavior can occur. Alternatively, differences in fatty acid oxidation related to exercise intensity may have an important role in altering glucose homeostasis, possibly via reductions in ectopic lipid deposition (Newsom et al., 2013). However, we did not see any changes in fasting plasma insulin or insulin sensitivity estimated by HOMA after training. The lack of change in HOMA is in agreement with some (Skleryk et al., 2013) but not all (Hood et al., 2011) short-term training studies. It could be that body composition changes are needed for exercise to improve insulin sensitivity (Houmard et al., 2004; Karstoft et al., 2013).

2.4.2.2 LPS stimulated whole blood cultures.

Previous studies examining MICT and/or resistance training have shown that reduced TLR4 is accompanied by reduced induction of cytokines in whole blood stimulated with LPS (Markofski et al., 2014; McFarlin et al., 2004; Timmerman et al., 2008). In the current study, reductions in TLR4 expression were not accompanied by changes in cytokine secretion following LPS-stimulation of whole blood cultures. This indicates that despite a reduction in cellular markers of inflammation, innate immune function has been maintained. A longer training may be required to detect changes in cytokine secretion (McFarlin et al., 2004). In addition, whole blood

culture supernatants were only collected at 24 hours of stimulation, which may not have been optimal for detection of differences in individual cytokines.

2.4.3 Limitations.

It is possible that the predominance of females in this study may have influenced the findings and this may explain the difference between our findings and previous findings that show an increase in monocyte TLR4 after two weeks of HIIT in males (Child et al., 2013). Previous studies have described differences in the inflammatory response to LPS stimulation (Asai et al., 2001) and glucose control response to HIIT (Gibala, Gillen, & Percival, 2014) between males and females. We did not have the statistical power to analyze potential sex differences; however, when only the female participants were analyzed, the results remained the same as when male and females were combined.

Although participants were told not to alter their diet during the course of the study we did not specifically control or measure dietary intake so it is possible that alterations in diet may have contributed to the observed changes in bodymass/BMI. However, the small changes in body mass/composition were equal between groups and likely of little clinical significance.

Our results indicate that over two weeks, both HIIT and MICT may have anti-inflammatory effects, however future studies are needed to determine how these exercise interventions compare over the long term. Changes to adipose tissue mass over prolonged training (12-16 weeks) may contribute to greater anti-inflammatory effects but this remains to be determined. The optimal exercise for far loss is currently unclear as some studies have indicated superior fat loss after HIIT

(Boutcher, 2011; Trapp, Chisholm, Freund, & Boutcher, 2008; Trapp et al., 2008) whereas others report greater fat loss after MICT (Keating et al., 2014; Nybo et al., 2010) or comparable changes between these types of training (Wallman, Plant, Rakimov, & Maiorana, 2009).

2.4.4 Summary.

In summary, short-term HIIT and MICT significantly improved VO_{2PEAK} and reduced monocyte and lymphocyte TLR4 expression and lymphocyte TLR2 expression in a group of inactive adults at risk of developing T2DM. These findings provide support that reductions in TLRs on multiple immune cell types are a possible direct anti-inflammatory response to short-term exercise training at either high or moderate intensity. Both HIIT and MICT reduced plasma fructosamine, providing evidence of improved glucose control. However MICT showed a significant reduction in fasting plasma glucose, not seen after HIIT, and a greater reduction in neutrophil TLR4, which may be attributable to longer exercise duration. More research is warranted to determine whether the direct anti-inflammatory effects seen at the cellular level are related to improving cardiometabolic health and reducing risk for T2D over time.

3.0 Discussion.

In this thesis, two weeks of both HIIT and MICT led to positive improvements in a number of cardiometabolic and inflammatory variables in previously inactive adults who were at elevated risk of developing T2DM. Although time-efficient HIIT was effective, contrary to the overarching hypotheses it was not superior to MICT for improving any of the parameters assessed in this short-term study. The following discussion will expand upon the findings from this thesis, place them into context in the literature, address their significance, and provide suggestions for future research.

3.1 Effect of HIIT and MICT on cardiorespiratory fitness.

The effects of two weeks of HIIT or MICT on cardiorespiratory fitness was measured and I hypothesized that HIIT would result in a greater increase in fitness as measured by VO_{2PEAK} (hypothesis 1). However, this hypothesis was not supported, as both HIIT and MICT resulted in equal improvements in fitness (5.85% after HIIT and 5.70% after MICT). My hypothesis was based on previous research as reviewed by (Weston et al., 2014), which indicates superior improvements in cardiorespiratory fitness after HIIT compared to MICT. A major difficulty in comparing HIIIT to MICT is matching the two conditions. This may be done through a combination of matching duration of exercise and/or exercise energy expenditure. However, matching the time spent exercising in MICT and HIIT removes the time efficient aspect of HIIT, which is one of the perceived benefits and major attractive features of this form of exercise training. Within this study we attempted to match the

conditions for external work but to maintain time-efficiency of HIIT the exercise duration was longer in MICT. However, caloric expenditure during each exercise session was not explicitly measured due to practical reasons in attempting to quantify energy expenditure for the different modes of exercise used (cycling, treadmill walking, elliptical and outdoor walking) and the non-steady-state nature of HIIT.

As this study design did not match duration of exercise in HIIT and MICT, this might have accounted for the similar improvement in VO_{2PEAK} between conditions. Studies that show superior improvements in VO_{2PEAK} after HIIT tend to use study designs where duration is matched to MICT (e.g., Karstoft et al., 2013; Weston et al., 2014). However, equal improvements in HIIT and MICT are more common when duration is not matched (Gibala et al., 2006; McKay et al., 2009; Rakobowchuk et al., 2008). When compared to previous studies employing a longer training period (6 – 16 weeks), HIIT results in equal (Burgomaster et al., 2008) or superior improvements of VO_{2PEAK} (Ciolac et al., 2010; Nybo et al., 2010; Tjonna et al., 2008), even when exercise sessions were not matched for duration. Thus it is possible that if this study were extended hypothesis 1 may have been supported. Considering this previous research I expect that in order for superior improvements in VO_{2PEAK} to occur after HIIT compared to MICT, both exercise protocols would need to be matched for duration and volume or the training intervention would need to be at least six weeks long.

Despite the fact that there was no difference between HIIT and MICT in improving cardiorespiratory fitness, this study indicates that two weeks of either type

of training is sufficient for significant improvements in cardiorespiratory fitness to be measured. Cardiorespiratory fitness is an important predictor of all-cause mortality, with moderate fitness having a protective effect against smoking, high cholesterol and high blood pressure (Blair et al., 1996). This finding has also been replicated in T2DM, with fitness being inversely correlated to all-cause mortality (Wei, Gibbons, Kampert, Nichaman, & Blair, 2000). An increase in cardiorespiratory fitness is an important contributor to improved health and reduced risk of all-cause mortality and the ability of short-term HIIT and MICT to increase this parameter is an important finding of this thesis.

3.2 Changes in metabolic markers

I hypothesized that HIIT would result in superior improvements in glucose control as measured by fasting glucose, insulin, HOMA-IR and HOMA-β scores (hypothesis 2); however, this hypothesis was not supported.

3.2.1 Change in fasting glucose.

After two weeks of HIIT there was no change in fasting glucose concentration; however, there was a significant decrease in fasting glucose after MICT. This was the opposite of what I had hypothesized. The potency of HIIT for improving glucose control has been shown after acute exercise bouts using continuous glucose monitoring (CGM) (Gillen et al., 2012; Little et al., 2014) and there has been evidence indicating that HIIT compared to MICT results in superior improvements in fasting plasma glucose in populations already diagnosed with insulin resistance or T2DM (Karstoft et al., 2013; Tjonna et al., 2008).

Despite these reported benefits of HIIT, exercise duration has been shown to be an important factor for improving insulin sensitivity and glucose control (Houmard et al., 2004). Within this study the HIIT group spent ~50% less time exercising compared to MICT. This is in opposition to the above-mentioned studies where HIIT resulted in superior improvements in glucose control (Karstoft et al., 2013), where both duration and volume of HIIT and MICT were matched. Thus, as with VO_{2PEAK}, if HIIT and MICT were matched for exercise duration, there may have been different results in regards to glucose control after HIIT compared to MICT, and HIIT may have been superior to MICT seen previously. To maintain the time-efficiency of HIIT for practical application it was decided not to match HIIT and MICT for exercise duration in this study.

One potential reason for the decrease seen in fasting glucose after MICT but not HIIT may be related to possible changes in epinephrine and cortisol release during exercise. With the higher intensity exercise in HIIT, there may be in an increased release of epinephrine and cortisol, both of which contribute to stimulating increased hepatic glucose output (Howlett, Febbraio, & Hargreaves, 1999). An acute study in trained athletes previously reported increased cortisol post-HIIT compared to MICT, accompanied by elevated plasma glucose after HIIT (Peake et al., 2014). In this study, fasting plasma glucose was measured up 72 hours following the final training bout so it is unlikely that glucose have remained elevated over this time course but the kinetics and timecourse of the glucoregulatory responses to HIIT and MICT were not assessed. Further studies measuring the acute and long-term

training effect of the heightened epinephrine response to HIIT and MICT may provide insight into the observed reductions in fasting glucose seen after MICT.

Differences in lipid metabolism during exercise may have played a role in the changes in fasting glucose observed after MICT but not HIIT. Lower intensity training as seen in MICT has been shown to increase lipid metabolism after both acute (Peake et al., 2014) and chronic training interventions (Venables & Jeukendrup, 2008). It is possible that the change in lipid metabolism at lower exercise intensities can result in changes in glucose control (Newsom et al., 2013). The mechanism for this is currently unclear but may be related to reduced accumulation of toxic lipid intermediates in skeletal muscle or liver (Schenk et al., 2009) or alterations in FFA availability (Newsom et al., 2013).

3.2.2 Change in fasting insulin.

There was no change in fasting plasma insulin after either HIIT or MICT following the two-week training intervention. While there have been some studies indicating that two weeks of training is sufficient for a reduction in fasting insulin to be observed (Hood et al., 2011; Whyte, Gill, & Cathcart, 2010), there are multiple other studies that indicate two weeks and even longer is not sufficient for this adaptation to occur (Karstoft et al., 2013; Keating et al., 2014; Skleryk et al., 2013).

One study employing 15 weeks of HIIT suggests that measurable changes in fasting plasma insulin are significantly correlated with reductions in adipose tissue of at least 10% or more (Trapp et al., 2008). Within our study the short duration of training and small change in body mass may have been a limiting factor in detecting a reduction in insulin levels.

There are a number of reasons why the effect of exercise training on insulin is mixed. Previous research has indicated that changes in peripheral insulin levels after exercise training may not be an accurate marker of changes in glucose control, as overall changes in the dynamics of insulin secretion appear to more important (Krotkiewski et al., 1985). Improvements in β-cell function, HbA1c and plasma glucose are not dependent on changes in fasting plasma insulin and the degree of change in insulin levels after training may be dependent on the initial level of β-cell function (Dela, von Linstow, Mikines, & Galbo, 2004). With this in mind and given the heterogeneous nature of our participants, it is perhaps not surprising that there were no systematic changes in fasting plasma insulin over the two week period.

3.2.3 Change in HOMA-IR and HOMA-%β.

There were no changes in HOMA-IR or HOMA-%β scores over the two-week period in either group. As the HOMA model is based on both changes in plasma glucose and insulin (Wallace, Levy, & Matthews, 2004) it is possible that over a prolonged training period improvements in insulin sensitivity and beta cell function, as measured by HOMA, may improve. This is supported by longer training interventions (8 – 16 weeks of training), which indicate that HIIT compared to MICT leads to greater improvements in HOMA scores (Earnest et al., 2013; Mitranun, Deerochanawong, Tanaka, & Suksom, 2014; Tjonna et al., 2008). Furthermore, transient improvements in insulin sensitivity may have been lost by post testing ~72 hours after the last exercise bout (Boule et al., 2005). This time point was chosen for blood sampling to remove the impact of the last bout of training in both groups, but it is possible that improvements in HOMA-IR would be seen if insulin was measured

closer to the final bout of training. For example, recent work from our lab has shown that a single bout of HIIT (similar to the protocol used in this study) results in an improvement in HOMA-IR measured ~24 hours after exercise in overweight/obese young females (Durrer et al., 2015).

3.3 Changes in TLR expression and circulating cytokines.

I hypothesized that HIIT would result in greater reductions in TLR as well as lead to a greater improvement in circulating pro-inflammatory cytokines when compared to MICT (Hypothesis 3). It was believed this would be mediated by a greater improvement in fitness and/or glucoregulation after HIIT. The results from this study do no support this hypothesis.

Prior to this study there was evidence to suggest that each bout of HIIT may result in a “pro-inflammatory” stimulus (Child et al., 2013; Zwetsloot, John, Lawrence, Battista, & Shanely, 2014); however, it is believed that over time this would lead to an adaptive anti-inflammatory response. It is still possible that after only two weeks, HIIT was still more “pro-inflammatory” rather than adaptive when compared to MICT. However, there was still a reduction in lymphocyte and monocyte TLR4 after HIIT, providing some evidence for an anti-inflammatory effect. Even though there was a reduction in TLR expression (i.e., a direct anti-inflammatory effect of exercise measured at the cellular level), there may be a delay between changes in cell surface expression and reduced markers of inflammation such as circulating cytokines.

3.3.1 Changes in Toll like receptors.

The significant decrease in TLR4 and 2 after two weeks of exercise provides evidence for a decrease of inflammation at the cellular level. TLRs have an important role in modulating chronic low-grade inflammation associated with metabolic disease thus reductions in their expression through exercise and physical activity has positive implications for long term health (Gleeson, McFarlin, & Flynn, 2006).

3.3.1.1 Changes in TLR4 expression.

There was a significant reduction in TLR4 expression on lymphocytes and monocytes, with no difference between HIIT and MICT. Additionally there was a significant reduction in TLR4 expression on neutrophils after MICT but not HIIT. These results are not in agreement with my hypothesis as I expected a greater reduction in TLR4 after HIIT compared to MICT. Reductions in TLR4 have been shown in previous after training interventions of both aerobic and resistance exercise of various duration (4 – 16 weeks) studies (Flynn et al., 2003; McFarlin et al., 2004; Stewart et al., 2005). Contrary to this, HIIT has been shown to increase TLR4 expression over a two-week period (Child et al., 2013). Therefore this is one of the first studies to show that HIIT reduces TLR4, despite the fact that MICT demonstrated a more potent anti-inflammatory effect, as measured by reductions of TLR4 on all three immune cell types measured.

There is currently limited literature investigating the mechanisms hypothesized for this post exercise reduction in TLR4. During exercise there may be

increased exposure to TLR4 ligands such as LPS, peptidoglycan, and double stranded RNA (Takeda & Akira, 2005) along with HSP70 (Rodriguez-Miguelez et al., 2014). In response to this exposure, there may be a down regulation of TLR4, in order to prevent over stimulation of pro-inflammatory cascades (i.e., immune tolerance). Without direct measurement of these proposed TLR4 ligands in response to each acute bout of HIIT or MICT throughout training I cannot conclude if exposure to these ligands is the mechanism resulting in the decrease in TLR after HIIT and MICT.

As previous research has shown a correlation between hyperglycemia and increased TLR4 expression and activation (Dasu et al., 2008; Dasu et al., 2010) it is possible that transient improvements in plasma glucose associated with exercise would contribute to the changes in TLR expression. This mechanism is partially supported by the reduction in plasma fructosamine (a marker of overall exposure to hyperglycemia over the preceding two weeks) following both HIIT and MICT. However, as TLR4 expression was reduced after both HIIT and MICT yet fasting glucose was only reduced after MICT, changes in glucose control would not appear to explain all of the changes in TLR4 expression.

How each bout of exercise throughout a training period, in HIIT or MICT, acts to influence changes in basal TLR expression that constitutes the adaptive response is still unknown. Further studies, especially acute and mechanistic studies examining whether immune tolerance responses can explain chronic training adaptation (Rodrigues-Miguelez et al. 2014; Takeda & Akira, 2005), are warranted.

3.3.1.2 Changes in TLR2 expression.

In agreement with previous literature there was little or no change in TLR2 after exercise in both HIIT and MICT (Child et al., 2013; Stewart et al., 2005). There was no significant change in TLR2 expression on monocytes and neutrophils after training but there was a significant decrease in TLR2 on lymphocytes, with no difference between the two groups. To my knowledge this is the first study to report an exercise-induced reduction in TLR2 on lymphocytes. Immune tolerance mechanisms similar to that of TLR4 (4.3.2.1) may also play a role in the regulation of changes in TLR2 on lymphocytes, but it is currently unclear as to how exercise might promote a reduction in lymphocyte TLR2.

It is also important to note that the amount of literature measuring changes in TLR4 on monocytes is greater than that examining changes in TLR2 (Stewart et al., 2005). This lack of literature may be due to earlier studies indicating no change in TLR2 and thus leading subsequent researchers devoting less attention to changes in TLR2 after exercise.

3.3.1.3 Importance of immune cell type

Although one previous study has measured TLR on immune cells other than monocytes by using peripheral blood mononuclear cells (PBMCs; a mixture of isolated cells containing primarily lymphocytes but also some monocytes) (Reyna et al., 2013), this study is the first to examine the effect of exercise on TLR expression on three immune cell types (lymphocytes, monocytes and neutrophils). Unlike Reyna et al (2013) who saw no change in TLR2 or 4 after 15 days of daily exercise, we detected distinct changes in expression on the various immune cell types. One

reason for the differences seen in this previous study may be due to the difference in methods used to measure TLRs. In our study, we used flow cytometry to measure TLR surface protein expression, whereas Reyna et al (2013) used western blot to assess total protein content. Flow cytometry is able to directly measure and quantify TLR surface protein on individual immune cells using fluorescence-conjugated antibodies whereas western blotting assesses total TLR protein in crude cell homogenates using less sensitive semi-quantitative chemiluminescence-based detection.

As neutrophils are the most abundant immune cells, the superior reduction in TLR4 expression on neutrophils after MICT but not HIIT may provide some evidence for increased anti-inflammatory benefits of MICT. Neutrophils are known to initiate immune responses and neutrophil TLR4 has been shown to mediate neutrophil survival and activation (Sabroe et al., 2003), thus it could be hypothesized that reductions in TLR4 may result in less downstream pro-inflammatory activation. The difference in neutrophil TLR4 expression after MICT may provide evidence for MICT being superior at reducing inflammation compared to HIIT and represents a key contribution to the exercise immunology literature from this thesis.

The vast majority of research within the field of TLRs and exercise has examined changes in TLR4 on monocytes; however, this research may have been missing a large component of the effect of exercise on the immune system as monocytes make up only 5-15% of the circulating immune cell population (Rogacev et al., 2010). Through measuring changes in TLRs on all three immune cell types we

have shown a direct and systemic anti-inflammatory effect of both HIIT and MICT that seems to be more pronounced after MICT.

3.3.2 Circulating cytokines.

It was hypothesized that HIIT would contribute to decreased basal circulating cytokines; however, this was not supported, as there was no change in pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) after training in either exercise condition. In addition, there was a significant reduction in plasma IL-10 (an anti-inflammatory cytokine) after MICT only.

Concentrations of circulating cytokines after training in populations with chronic disease and inflammation have been shown to decrease (Goldhammer et al., 2005). However, training studies in both healthy active and inactive populations have shown plasma that cytokines remain the same (McFarlin et al., 2004; Stewart et al., 2007; Zwetsloot et al., 2014). While we expected our participants to respond like the former group, our results were more in agreement with that of a healthy population.

The measurement of cytokines within circulating plasma is dependent on excess spillover of cytokines from immune cells (Womack et al., 2007), adipose tissue, vasculature, liver and skeletal muscle (Lumeng & Saltiel, 2011). Although fitness/physical activity levels do appear to correlate to the concentration of pro-inflammatory cytokines (Reuben, Judd-Hamilton, Harris, & Seeman, 2003; Toft et al., 2002), they do not appear to be directly modulated by training interventions in healthy populations. This highlights the importance of measuring changes of other inflammatory markers at the cellular level, such as TLRs, because measurements of

cytokines within the circulation may not reflect or detect exercise-induced anti-inflammatory changes at the cellular/molecular level. While there was no change in basal circulating cytokines, transient changes after each exercise bout may still have contributed to overall reductions in systemic inflammation (Petersen & Pedersen, 2005).

IL-10 is an anti-inflammatory cytokine (Joyce et al., 1994) and one may think it would increase after training (indicating a more “anti-inflammatory” environment) there was a decrease in IL10 levels after MICT. However, this unexpected decrease after MICT may be a marker of anti-inflammatory processes occurring and not pro-inflammatory ones. IL-10 is known to increase in response to inflammation, so elevated levels of IL-10 may be a compensatory response to chronic low-grade inflammation (Avdiushko et al., 2001). Thus, it could be speculated that the anti-inflammatory effects of MICT (i.e., greater reduction in neutrophil TLR4 and fasting glucose) reduced the stimulation of IL-10, leading to the observed decrease after training. While the difference between IL-10 for HIIT and MICT at baseline was not statistically significant, MICT tended to have a higher circulating concentration of IL-10 (7.7 ± 11.6 pg/ml in MICT vs. 2.4 ± 2.9 pg/ml in HIIT). This apparent discrepancy was driven largely by one individual in MICT who had a plasma IL-10 concentration of 45 pg/ml at baseline. IL10 concentration in this individual remained similarly high post-training and this data point was kept in for statistical analysis. When this value was removed, the average fasting plasma IL10 was reduced to 5.0 ± 5.1 pg/ml in the pre-training state and 3.7 ± 4.2 pg/ml post-training for MICT and there was still a

significant reduction following training (p=0.03). Therefore based on the data baseline differences do not appear to explain the reduction in IL10 seen after MICT.

3.4 Changes in cytokine production after whole blood stimulation.

In concordance with previous hypotheses regarding the anti-inflammatory effect of HIIT compared to MICT, I hypothesized that HIIT would result in a greater reduction in cytokine release in stimulated *ex vivo* whole blood cultures (hypothesis 4). This hypothesis was not supported as there was no change in stimulated whole blood culture cytokine production after training in either group. Measuring the cytokine production allows for the functional ability of the immune cells to be assessed before and after training but this technique is not without its limitations.

3.4.1 LPS stimulation stimulated cytokine production.

LPS is a TLR4 agonist, which stimulates TLR4 activation and subsequent cytokine release. There was no change in cytokine production after LPS-stimulation in either group after the intervention, which is in agreement with some previous research (Stewart et al., 2005) but contradictory to others (Phillips et al., 2001; Timmerman et al., 2008). This lack of change in cytokine production may be due to the length of the training intervention as previous work showing a decrease in cytokine production used training interventions of at least 10 weeks in length (Phillips et al., 2001; Timmerman et al., 2008). Although Timmerman et al (2008) also note that pre and post testing in their study coincided with early summer to early fall, which happen to also represent the time points of a seasonal depression in LPS stimulation (Myrianthefs et al., 2003), potentially exaggerating the training induced decreases.

While it would be expected that along with a decrease in TLR4 expression there would also be a decrease in LPS/TLR4-mediated cytokine production (Flynn et al., 2003), it is possible that due to the nature of the whole blood culture technique (i.e., multiple cell types interacting to dictate the cytokine secretion response) and the heterogeneous population of participants studied that no change was seen. In addition, due to feasibility issues whole blood cultures were only prepared for 15 participants in HIIT and 10 in MICT with supernatants collected at one time point (24 hours) only. This could have limited the ability to detect changes in cytokine secretion. Alternatively, it could be argued that similar cytokine secretion is evidence that the innate immune response to a bacterial pathogen is preserved after exercise training, despite a down regulation in TLR4 expression. Further investigation of LPS-induced cytokine production between the high and the low TLR4 expressers within our sample population may tease out any changes after exercise.

3.4.2 PAM-CSK4 stimulated cytokine production.

Whole blood was stimulated with the TLR2 ligand PAM-CSK4 in order to measure functional changes in TLR2 activity. There were no changes in TLR2-induced cytokine production after either HIIT or MICT. Since there was a small change in lymphocyte TLR2 only after training, the lack of change in PAM-CSK4-stimulated cytokine production was not surprising. Previous research using peptidoglycan (PGN) stimulation of TLR2 also showed no change after training (Stewart et al., 2005). While PGN and PAM-CSK4 are different molecules, they are both TLR2 ligands and therefore it would be expected that TLR2 would respond similarly to both.

Research from our lab has shown an increase in TLR2 activation via PAM-CSK4 incubation after one week of a high-fat diet, which appeared linked to increases in circulating FFA (Wan et al., 2014). Thus it is possible that TLR2 is more sensitive to changes in circulating FFA, which did not change within this two-week training intervention.

3.4.3 Rationale for whole blood culture

There are multiple models of immune cell culture available. As mentioned earlier, PBMCs are one common method but there are limitations to this technique as there is an unknown ratio of monocytes to lymphocytes (De Groote et al., 1992). As we measured TLR expression on multiple cell types, we also wanted to measure TLR function on the same immune cell types. By using whole blood rather than PBMCs, the whole blood culture results should reflect the cumulative response of monocytes, neutrophils and lymphocytes. By stimulating whole blood rather than isolated cell types, in which there is a distinct lack of immune and humeral factors normally found in whole blood (Albers et al., 2005), the results remain more applicable to whole body physiological situations (Damsgaard, Lauritzen, Calder, Kjær, & Frøkiær, 2009).

However whole blood cultures can be criticized because there is a lack of specificity regarding which cell types are producing the measured cytokines. Despite being validated as an accurate and reliable measure of cytokine production (Damsgaard et al., 2009), assessing the individual responses of isolated cells separately would have represented a more reductive approach to characterizing the direct effects of exercise on the function of different immune cells.

3.5 Changes in anthropometrics.

3.5.1 Body mass and BMI.

Two weeks of HIIT and MICT significantly reduced body mass and BMI, with no difference between groups. This was not expected as the two week training intervention was chosen to minimize any changes in body mass and BMI to measure the direct effects of exercise on chronic low-grade inflammation without the influence of changes in adipose tissue. However, the changes in body mass and BMI were small (average reduction of 1.8 ± 1.5%) which is likely of little clinical or physiological significance. Future studies within our lab will now include Dual-energy X-ray absorptiometry (DEXA) scanning to confirm changes in body composition that cannot be measured by body mass alone (e.g., fat mass, visceral adipose tissue mass). This technique was not available at the time of data collection for this study so unfortunately detailed analyses of body composition could not be conducted.

As this change in body mass and BMI was less than 5%, it was unlikely to be clinically significant (Blackburn, 1995). Additionally changes in body mass were not correlated to changes in TLRs (*appendix 2.7*) providing further support that the significant changes in immune markers after training were a reflection of the direct effect of exercise and not due to reductions in adipose tissue.

3.5.2 Blood pressure.

There was a main effect of time with no difference between the groups for systolic blood pressure and mean arterial pressure after two weeks of training (*appendix 2.1*). There were no significant changes in diastolic blood pressure after HIIT or MICT (*appendix 2.2*). However, without more regular blood pressure

measurements it is not possible to confirm this as a true effect of training. At post-testing it is likely that participants generally feel more comfortable about being tested and working with the research assistants and therefore have lower anxiety. This may contribute to a lower blood pressure response when compared to pre-testing. A non-training control group would have been needed to confirm these reductions in blood pressure were true training effects.

3.6 Limitations

With only two intervention groups (HIIT and MICT) there was limited ability to determine if differences in the outcome measures were related to the difference in exercise intensity or duration. In order to examine the relationship between exercise intensity and duration on the outcome measures, a third group consisting of time matched HIIT would be required. This is discussed in further detail under future work (5.1).

The training intervention was designed for each session of HIIT and MICT to be matched for energy expenditure (appendix 1.9). However, actual caloric expenditure would have been different for each participant within the study. While in steady state exercise HR may be used as a simple tool in order to estimate metabolic workload and this is an estimate of VO_2. Although this remains a valid assumption for steady state exercise, it may not be valid when applied to HIIT due to time lags in HR elevation during the intense intervals and HR lowering during recovery intervals when compared to VO_2 (energy cost). For this reason it is always difficult to match energy expenditure between HIIT and MICT. For this study we decided that in order to maintain the time efficient aspect of HIIT, the two conditions

would be matched for estimated energy expenditure only and not duration of exercise. This resulted in the MICT group exercising for nearly twice as long as HIIT.

As mentioned earlier, a major impetus for conducting this short-term exercise training study was to minimize changes in body mass in order to measure the direct effects of exercise without interference of changes in adipose tissue mass. However, there was a small yet statistically significant reduction in body mass and BMI after both HIIT and MICT. It is possible that these reductions confounded the direct effects of exercise on inflammation and metabolic markers but since changes in body mass were not significantly correlated to changes in immune and metabolic parameters it is unlikely that the small reductions had an effect on our results (*appendix 2.7*).

The significant reduction in body mass could be due to a lack of explicit diet control, which is another limitation of this study. Over the two-week period, dietary intake was not specifically measured or controlled. A systematic change in diet resulting in a reduction in body mass would not have been expected over this short period but it is possible that after enrolling in the study subjects could have been motivated to change other aspects of their lifestyle, including diet, which may have contributed to the small weight loss. If this were the case, it would seem unlikely that this would have differed between groups, which is supported by the similar degree of minor weight loss seen in both HIIT and MICT.

Post fasting blood samples were obtained 48-72 hours after training. This time point was chosen to remove any acute carryover effects of the last bout of exercise, and aimed to provide a more accurate measure of any training adaptations

that occurred over the two-week period. However, measuring at multiple time points immediately after training as a time course of the acute response of HIIT versus MICT may have added insight into metabolic and immunomodulatory responses of the two exercise modes. Previous research has indicated prolonged improvements in metabolic markers (fasting glucose and HOMA score) up to 72 hours post the last exercise bout in HIIT and not MICT (Earnest et al., 2013); however, this was not replicated within this thesis. The inflammatory response to an acute bout of HIIT is not altered by two weeks of training in healthy individuals (Zwetsloot et al., 2014). However, as the immune response may be different in those at elevated risk of developing T2DM, a similar study that utilizes a time course of the training effect on immune markers after HIIT and MICT would still be warranted.

4.0 Future research, significance, and conclusions

4.1 Future research

As mentioned above under limitations (4.6) the design of this study compared a time efficient HIIT protocol to an energy expenditure matched MICT protocol, resulting in the duration of each training session not being matched and on average the duration of MICT was double that of HIIT. This design, while highlighting the time efficient nature of HIIT, does not allow for the determination of differences in cardiorespiratory fitness, inflammation and metabolic control to be attributed to exercise intensity or duration. In order to do this a third experimental group would have to be added; a HIIT protocol that is duration matched to MICT. To extend the HIIT protocol without drastically increasing caloric expenditure a longer warm up and cool down at a very light intensity could be added and the number of intervals reduced to maintain equal energy expenditure. By having the three experimental conditions (and a non-exercise control group) changes in all parameters could be defined by exercise duration in addition to mode. A study with two exercise arms has been conducted, with duration and mean intensity matched MICT and interval walking (Karstoft et al., 2013). This study found that when duration and energy expenditure was matched, HIIT was superior in improving cardiorespiratory fitness and glycemic control (Karstoft et al., 2013). While this study supports the superior improvements after interval training it does so at the detriment of the time efficient nature of HIIT.

In this thesis I aimed to maintain body mass and BMI over a short-term intervention to examine the direct effects of exercise without reductions in adipose

tissue. However this does not lend itself to long-term applications. An extension of this study could be conducted to see if the effects of HIIT and MICT remain the same in the long term. HIIT has been shown to result in greater reductions in adipose tissue when compared to MICT (Boutcher, 2011) and because of this I would hypothesize that over a longer intervention period there would superior improvements in inflammation and glycemic control in HIIT compared to MICT. Future long-term studies are warranted to test and extend this hypothesis.

4.2 Significance of findings

While there have been multiple studies at comparing the effects of HIIT and MICT on improving cardiorespiratory fitness (Weston et al., 2014), there is limited research examining the direct effects of these two exercise modes on markers of metabolic health and inflammation in previously inactive adults at increased risk of developing T2DM. This thesis indicates, through reduced TLR4 expression on lymphocytes, monocytes and neutrophils, 2 weeks of exercise can have a direct and systemic effect on reducing markers of cellular inflammation. This provides further evidence for reductions in TLR to be a main mechanism through which exercise acts to reduce chronic low-grade inflammation. While previous studies have focused on the changes in TLR on monocytes only (Flynn et al., 2003; McFarlin et al., 2006; Stewart et al., 2005), this is the first study to examine the changes in TLR2 and 4 in three distinct immune cell populations (monocytes, lymphocytes and neutrophils).

Overall, the findings provide further evidence for time efficient HIIT to have equal cardiorespiratory benefits compared to MICT as well as reinforcing that previously inactive adults can perform HIIT and accrue benefits. However, based on

the greater impact of MICT for reducing neutrophil TLR4 and fasting glucose it may be that traditional MICT, which involved exercise of a longer duration than the time-efficient HIIT protocol used in this study, may be advantageous for reducing certain markers of cellular inflammation and metabolic health.

4.3 Conclusion.

In conclusion this thesis indicates that two weeks of either HIIT or MICT significantly improved cardiorespiratory fitness. However, while both HIIT and MICT reduced TLR4 expression on monocytes and lymphocytes, only MICT resulted in a reduction in neutrophil TLR4 and a lowering of fasting blood glucose. There were no significant changes in fasting pro-inflammatory cytokines, insulin resistance, or FFA. Overall, these results do not support the original hypothesis that HIIT would be more effective at reducing inflammation and improving cardiometabolic health in previously inactive adults at increased risk of developing T2DM but this study provides some evidence for a direct and systemic anti-inflammatory effect of both HIIT and MICT. Whether exercise induced anti-inflammatory changes at a cellular level are related to improved health and decreased risk of T2DM over the long-term remains to be seen and further research examining this is warranted.

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Appendix A: Expanded methods.

A.1 Glucose protocol for the Chemwell 2910

Assay principle:
This assay is an enzymatic endpoint reaction, based on the phosphorylation of glucose. Specifically glucose is phosphorylated by hexokinase (HK) forming glucose-6-phosphate (G-6-P). The G-6-P is oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) to produce NADH. The absorbance of the solution is read at 340nm, as a result of the increased NADH, which is directly proportional to the concentration of glucose in the sample.

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{HK} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + NAD^{+} \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \text{NADH} + H^{+}$$

Materials needed:

- Fresh plate, strip wells are in the cupboard above the Chemwell.
- Glucose hexokinase reagent: Stored in 4°c fridge. Pointe Scientific G5717-120 Glucose.
- Pointe scientific Chemistry Calibrator G7506 –50 Glucose. 177 mg/dl. Reconstitute with 5.0 ml of diluent added to the lyophilized serum. Gently invert intermittently over a period of 20 min. Immediately prior to use invert 5 – 10 times. Pipette 300 ul in to glass tube.
- 300 ul of 0 calibrator - 0.9% sodium chloride, in a falcon tube in the 4°c fridge door. Pipetted into glass tube.
- Pointe Scientific Level I (89±9 mg/dl) and II (299 ±30 mg/dl) controls. G7592-100 Glucose. Reconstitute with 5.0 ml of milliQ water added to the control. Replace the stopper and allow to sit for 10 min. Gently invert 3 times and swirl till homogenous.
    - Pipette 300 ul in to a glass tube.
- 100 ul aliquots of samples to be analyzed in 1.5 ml tubes. Spin down for 15 min at 1500 G at 4°C. if already in 100 ul samples then no need to do this.

Start up routine:

- Turn on the computer with software and open Chemwell manager.
  USER: ADMIN
  Password: admin
- Turn on the 2910 at the back.
- The start up check will run automatically – check that all systems pass, with green ticks – and press ok.

- Make sure program is in Chemistry mode – changed under the "Management" tab. Temp will be 37 degrees. It may take time for the plate to warm up to this temperature.
- Empty, wash and refill the bottle attached to the syringes with fresh milliQ water. This is to be done before each testing session.
- Run the start of day protocol – under the "routine" tab. During this check for bubbles in the syringe, if there are bubbles prime the syringes with 95% EtOH (can prime with MeOH) and then repeat the start of day protocol with fresh milliQ water.

Running the assay:

Calibration:

- This is the second tab along the bottom of the screen.
- In the top pull down menu under "Choose an assay…" Click on *Glucose – control out – expired*.
- On the top of the right hand panel there is an "Add Calibration" drop down menu. Pick [all] which will give you calibrator 0 and 2 and control 1 and 2.
    - For each fill in the required replicate number, normally 2 for all.

Loading Sample:

- Third tab along is sample. Click on this.
- Click on "Add sample ID" in the bottom left corner. You can either search for the participant ID already entered or add new by clicking EDIT.
- When you have all the sample ids you need highlight so that they are in blue and click CHOOSE. Click CLOSE to exit the popup box.
- The sample ids you chose should now be listed in the left hand column.
- In the middle column, click the Glucose assay. Click "ADD TEST" in the middle of the screen.
- Sample IDs should show up on the work list, the right panel. Pick number of reps for each sample = 2.
- Click "request" This will load both controls and samples.

Rack set up:

The layout tab will tell you the rack set up plus how much is required of each solution.

Rack one

- Position one = Glucose Hex Reagent – In a small round bottle.

Rack two

- Position one = Glucose calibrator level 0 (500 ul)
- Position two = Glucose calibrator level 2 (500 ul)
- Position three = Control I (500 ul)

- Position four = Control II (500 ul)
- The rest of the rack will be for your samples. Put them in the correct places with the tube lids cut off.

PRESS START RUN.
During the running of the samples the glucose hex reagent may run out. In this case pull out and refill. If an error of low fluid detected in a sample, highlight it under the "test list" tab and click RERUN.
The protocol is set up as follows.

- Add glucose hexokinase reagent, 297ul.
- Add sample, 3ul.
- Incubate 4min
- Read with primary filter, 340nm, and differential filter, 630nm.

Reviewing results
Once the test has finished a pop up box will tell you. Press "OK".
Check that the calibration passed (all in blue) and the controls were in range (Control 1 = 80 – 98 and control 2 = 269 - 329). You can check the curve too and the report for CVs.

On completion of assay:
Check results.
Select all and click "accept" If you do not do this the results will not transfer into the report tab.
Once under the report tab and results have shown up (you may need to search for them using the date and the glucose assay) you can click "export" and save as an excel spreadsheet.

END OF DAY
Run the "end of day" routine which is under the routines tab.

- Use 5% fresh bleach (i.e. 1:20 Bleach and milliQ water)

A.2 Non esterified fatty acid (NEFA) protocol on Chemwell 2910

Assay principle:
NEFA concentration in plasma is measured by this assay via an enzymatic colourimetric method. Acylation of NEFA by acyl-CoA synthetase (ACS) produces acyl-CoA. Acyl-CoA is then oxidized by acyl-CoA oxidase (ACOD) producing hydrogen peroxide (H_2O_2). The H_2O_2 reacts with 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) and 4-aminoantipyrine to form a purple pigment, which is measured at an absorbance of 550nm. This is proportional to the concentration of NEFA in the sample.

$$\text{RCOOH + ATP + CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA + AMP + PPi}$$

$$\text{Acyl-CoA + } O_2 \xrightarrow{\text{ACOD}} \text{2,3-trans-enoyl-CoA + } H_2O_2$$

$$2\ H_2O_2 + \text{MEHA + 4-aminoantipyrine} \xrightarrow{\text{POD}} \text{purple condesation product + 2 } H_2O$$

POD = peroxidase

Materials needed.

- Fresh plate, strip wells in the cupboard above the Chemwell.
- NEFA reagent A – prepared as per following instructions: Add one bottle of Solvent A (WAKO 995-34791) to one vial of Colour reagent A (WAKO 999-34691). Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for 1 month at 2-10 degrees.
- NEFA reagent B – prepared as per following instructions: Add one bottle of Solvent B (WAKO 993-35191) to one vial of Colour reagent B (WAKO 991-34891). Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for 1 month at 2-10 degrees.

NEFA standard solution (WAKO 276-76491) made up as follows with milliQ water. Pippette 300ul of each standard into a labeled glass tube. (at least 300 ul)

Standard #	FFA (mM)	Stock (ul)	H_2O (ul)
1	1.0	Undiluted	0
2	0.8	1000	250
3	0.5	500	500
4	0.25	250	750
5	0.1	100	900
6 Blank	0	0	1000

- 100 ul aliquots of sample – centrifuge 10min @ 3000 rev @ 4 degrees.

Start up routine:

- Turn on the computer with software and open chemwell manager.

USER: ADMIN

Password: admin

- Turn on the 2910 at the back.
- The start up check will run automatically – check that all systems pass, with green ticks – and press ok.
- Make sure program is in Chemistry mode – changed under the "Management" tab. Temp will be 37 degrees. It may take time for the plate to warm up to this temperature.
- Empty, wash and refill the bottle attached to the syringes with fresh milliQ water. This is to be done before each testing session.
- Run the start of day protocol – under the "routine" tab. During this check for bubbles in the syringe, if there are bubbles prime the syringes with 95% EtOH (can prime with MeOH) and then repeat the start of day protocol with fresh milliQ water.

Running the test:

Calibration/standard curve:

- This is the second tab along the bottom of the screen.
- In the top pull down menu under "Choose an assay…" Click on *WAKO NEFA– expired*.
- On the top of the right hand panel there is an "Add Calibration" drop down menu. Pick [all] which will give you Standard, 2, 3, 4, 5 and Blank.
- For each fill in the required replicate number. All = 2 each. Select "request" bottom right hand corner.

Loading Sample:

- Third tab along is sample. Click on this.
- Click on "Add sample ID" in the bottom left corner. You can either search for the participant ID already entered or add new by clicking EDIT.
- When you have all the sample ids you need highlight so that they are in blue and click CHOOSE. Click CLOSE to exit the popup box.
- The sample ids you chose should now be listed in the left hand column.
- In the middle column, click the WAKO NEFA assay. Click "ADD TEST" in the middle of the screen.
- Sample IDs should show up on the work list, the right panel. Pick number of reps for each sample = 2.

Rack set up:

The layout tab will tell you the rack set up plus how much is required of each solution.

Rack one

- Position one = NEFA color A
- Position two = NEFA color B

Rack two

- Position one = NEFA Standard (1mM)

- Position two = NEFA Stand 2 (.8mM)
- Position three = NEFA stand 3 (.5mM)
- Position four = NEFA stand 4 (.25mM)
- Position five = NEFA stand 5 (.1mM)
- Position six = NEFA blank
- The remaining positions will be for the samples. Put them in the correct places with the tube lids cut off.

PRESS START RUN.
During the running of the samples the colour A or B reagent may run out. In this case pull out and refill. If an error of low fluid detected in a sample, highlight it under the “test list” tab and click RERUN.
The protocol is set up to go through the following steps:

- Add sample – 4.5 ul
- Add Reagent A – NEFA color A – 180 ul
- Incubate 5 min
- Read on primary filter 545nm
- Reagent B – NEFA color B
- Incubate for 5min
- Read on primary filter 545nm

On completion of assay:
One the test has finished a pop up box will tell you. Press “OK”.
Check that the standards are all within range of their concentrations (all in blue). You can check the curve too and the report for CVs.
Select all and click “accept” If you do not do this the results will not transfer into the report tab.
Once under the report tab and results have shown up (you may need to search for them using the date and the glucose assay) you can click “export” and save as an excel spreadsheet.

END OF DAY
Run the “end of day” routine which is under the routines tab.

- Use 5% fresh bleach (i.e. 1:20 Bleach and milliQ water)

A.3 Insulin ELISA protocol on Chemwell 2910

Assay principle:
An enzyme-linked immunosorbent assay (ELISA) uses at least one antibody to bind to the specific molecule that is to be measured.
Specifically the Mercodia Insulin ELISA is a solid phase two-site assay, using a sandwich technique. The sandwich technique refers to the fact that the insulin is "sandwiched" between two anti-insulin antibodies, one immobilized to the bottom of the assay plate and second of which is added in solution and is perioxidase-conjugated. Excess anti-insulin antibodies are washed away and when the detection reagent 3,3',5,5'-tetramethylbenzidine (TMB) is added it reacts with the conjugate enzyme, resulting in a yellow product. The reaction is stopped by adding sulfuric acid and the colormetric endpoint can be read spectrophotometrically.

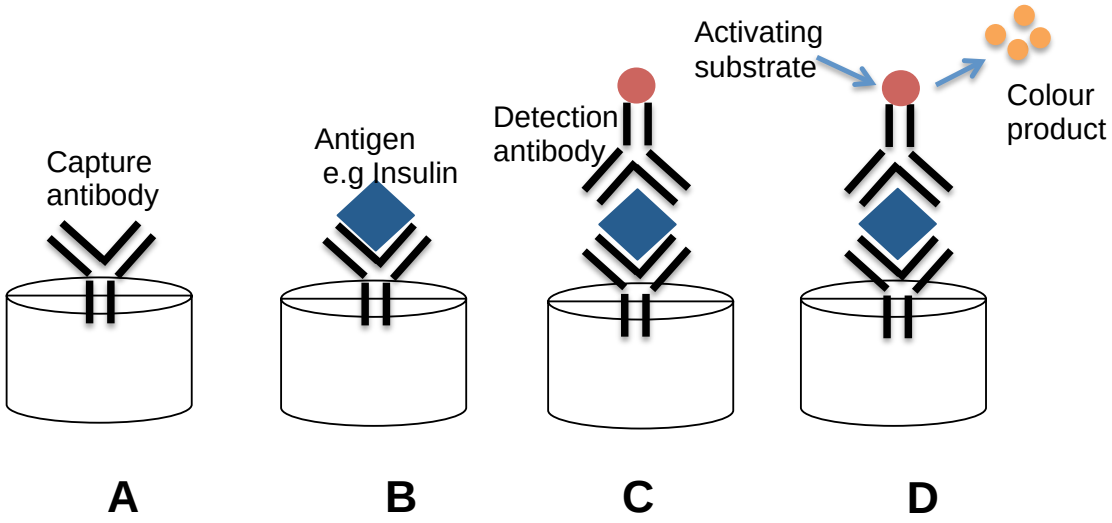


Figure 7 Sandwich ELISA technique. Capture antibody is immobilized on the bottom of the well (**A**), the sample is added to well and the antigen binds to the capture antibody (**B**). Peroxidase-conjugated detection antibody added and binds to antigen (**C**). An activating substrate is added (e.g TMB) reacting with the detection antibody to produce a colour change (**D**).

Materials needed:

- Mercodia insulin ELISA kit (10-1113-01).
  - Make up wash and place in wash bottle of Chemwell – for 1 plate = 35 ml of wash buffer to 700 ml of distilled water.
  - Make up Enzyme conjugate = 1.0 ml of Enzyme conjugate 11x and 10 ml of Enzyme conjugate buffer. Place in 30 ml bottle.
  - Place 20 ml of Substrate TMB into 30 ml bottle covered with tinfoil – light sensitive.
  - Place 5 ml of Stop solution into 30 ml bottle.
  - Pipette 100 ul each of standard 0,1,2,3,4,5 into labeled microtubes
- 1M HCL – found in Acids bin in lab. Need 20 ml placed into a 30 ml bottle.

- 4 x 30ml bottles for chemwell for enzyme conjugate, substrate TMB, stop solution and HCL.
- Lyphochek immunoassay controls 1,2, 3 (Bio-Rad 370). Made up by adding 1 mL of milliQ water, swirl gently and allow to sit at room tempture for 20 min. Mix gently again prior to use. Use 100 ul pipetted into labeled microtubes.

Start up routine:

- Turn on the computer with software and open chemwell manager.

  USER: ADMIN

  Password: admin
- Turn on the 2910 at the back.
- The start up check will run automatically – check that all systems pass, with green ticks – and press ok.
- Make sure program is in EIA mode – changed under the "Management" tab. Temperature will change to room temperature.
- Empty, wash and refill the bottle attached to the syringes with fresh milliQ water. This is to be done before each testing session.
- Run the start of day protocol – under the "routine" tab. During this check for bubbles in the syringe, if there are bubbles prime the syringes with 95% EtOH (can prime with MeOH) and then repeat the start of day protocol with fresh milliQ water.

Running the test:

Calibration and load samples:

- This is the second tab along the bottom of the screen.
- In the top pull down menu under "Choose an assay…" Click on *Mercodia Insulin – expired*.
- On the top of the right hand panel there is an "Add Calibration" drop down menu. Pick [all] which will give you standard 0,1,2,3,4,5 and Lyphochek 1,2, 3. Fill in the required replicate number = 2 each for standards and controls.

Loading sample

- Click on the sample tab – third along.
- Click on "Add sample ID" in the bottom left corner. You can either search for the participant ID already entered or add new by clicking EDIT.
- When you have all the sample ids you need highlight so that they are in blue and click CHOOSE. Click CLOSE to exit the popup box.
- The sample ids you chose should now be listed in the left hand column.
- In the middle column, click the Mercodia Insulin assay. Click "ADD TEST" in the middle of the screen.
- Sample IDs should show up on the work list, the right panel. Pick number of reps for each sample = 2.
- Click "request" this will take you to the rack set up lab.

Rack set up:

The layout tab will tell you the rack set up plus how much is required of each solution.

Rack one
- Position one = Insulin enzyme conjugate
- Position two = Insulin substrate
- Position three = Insulin stop solution
- Position four = hydrochloric acid.

Rack two
- Position one = Insulin standard 0
- Position two = Insulin standard 1
- Position three = Insulin standard 2
- Position four = Insulin standard 3
- Position five = Insulin standard 4
- Position six = Insulin standard 5
- Position seven = Lyphocheck 1
- Position eight = Lyphocheck 2
- Position nine = Lyphocheck 3
- The rest of the rack will be for your samples. Put them in the correct places with the tube lids cut off.

PRESS START RUN.
During the running of the samples reagents may run out. In this case pull out and refill. If an error of low fluid detected in a sample, highlight it under the “test list” tab and click RERUN.
The protocol is set up to run as follows:
- Add sample (25ul)
- Add insulin enzyme conjugate (100ul)
- Incubate 1 hour
- Wash 6 times
- Add insulin substrate (200ul)
- Incubate 15 min
- Add insulin stop solution (50ul)
- Mix for 10 seconds
- Read on primary filter 450nm and differential filter 630nm.

On completion of assay:
Check results.
Select all and click “accept” If you do not do this the results will not transfer into the report tab.
Once under the report tab and results have shown up you can click “export” and save as an excel spreadsheet.

END OF DAY
Run the “end of day” routine which is under the routines tab.
- Use 5% fresh bleach (i.e. 1:20 with bleach under the sink)

A.4 Calculations for HOMA-IR and HOMA-β

Homeostasis model of assessment estimate of insulin resistance (HOMA-IR) was calculated using the following equation:

$$\text{HOMA-IR} = \frac{\text{fasting plasma insulin x fasting plasma glucose}}{22.5}$$

Homeostasis model of assessment estimate of beta cell function as a percentage (HOMA-β) was calculated using the following equation:

$$\text{HOMA-}\beta = \frac{\text{20 x fasting plasma insulin}}{\text{fasting plasma glucose} - 3.5}$$

A.5 Toll-like receptor (TLR) determination protocol.

Assay principle

TLR 2 and 4 expression on immune cells (lymphocytes, monocytes and neutrophils) was measured using flow cytometry. Flow cytometry employs laser light scattering and fluorescence detection in order to measure cell number and characteristics within a moving stream of fluid.

The size and granularity of the cell can be determined by the way the light refracts off the cell as it passes through the laser. Cell size is determined by how the light is scattered, as detected by a receptor in the forward direction. Larger cells will refract more light and a higher forward scatter will be measured. The refraction of the laser off the cell is also detected at a right angle to the original laser beam. This is known as side scatter and gives an indication of the internal density or complexity of cell. For example a granulocyte will have higher side scatter due to the increased density ("granularity") of the cell. In conjunction with this fluorescence-conjugated antibodies are used to allow labeling of surface markers specific for cell types and/or receptors. The lasers excite the fluorochromes on the antibodies and the emitted light is detected, which is in proportion to the amount of the antigen present on the cell. A multi-colour fluorochrome-conjugated antibody panel can be designed and a gating strategy is employed to determine the median fluorescence intensity for each surface marker (TLR2 or TLR4) on the specific cell types based on scatter profile and fluorescence intensity of the different detector channels, which are specific for each fluorochrome.

Materials Needed

- 75mm sterile culture tubes, pipettes and pipette tips (sterile)
- Monoclonal antibodies with conjugated fluorochrome.
  - CD45 APC-Vio770 (Miltenyi)
  - CD14 Vioblue (Miltenyi)
  - TLR2 PE (Miltenyi)
  - TLR4 APC (Milteny)
  - CD15 FITC (Miltenyi)
- FcR Blocking Reagent (Miltenyi) to inhibit non-specific antibody binding to the Fc receptor of the cell.
- Propidium Iodide Solution (Miltenyi) stains dead cells.
- MACSQuant Running buffer (Miltenyi)
- MACSQuant Analyzer 10
- MACSQuant Calibration Beads to calibrate laser settings.
- 200ul of whole blood kept on ice and collected within 45min of starting the protocol.

Start up Routine

- Turn on the flow cytometer and login.
- Set the flow cytometer to acquisition mode

    - In the top right corner of the screen there is a button with the power symbol on it, press it and select “acquisition mode” from the menu that pops up
- Allow for at least 30mins for the flow cytometer lasers to warm up, after which a calibration can be run.

Calibration

- On the top menu bar of the flow cytometer, press the button with the barcode symbol on it, a red barcode scanner will start flashing through a small glass panel under the acquisition needle
- Hold the bottle of MACSQuant calibration beads in front of the scanner so that the barcode on the bottle is facing the scanner
- When the bottle has been scanned successfully, the scanner should flash green and an on-screen pop-up should appear. Follow the prompts from the on-screen pop-up
    - It will tell you to put one drop of the calibration beads into a 12x75mm round bottom tube, place the tube in the single tube holder, and press continue.
- The flow cytometer will now automatically calibrate itself.

Preparing the Blood Sample

- Label 3 12x75mm sterile round bottom tubes with subject number (on all of them) and either “Full Stain”, “FMO –TLR2”, and “FMO –TLR4”.
- Pipette 54ul of whole blood into the “Full Stain” tube and 6ul of FcR blocking reagent.
- Pipette 63ul of whole blood into the “FMO –TLR2” tube and 7ul of FcR Blocking Reagent
- Pipette 63ul of whole blood into the “FMO –TLR4” tube and 7ul of FcR Blocking Reagent
- Incubate all the tubes in the dark at 4°C for 10mins
- Following incubation, add 10ul of CD45, CD14, CD15, TLR2, and TLR4 to the “Full Stain” tube
- Add 10ul of CD45, CD14, CD15, TLR4, and MACSQuant Running buffer to the “FMO –TLR2” tube
- Add 10ul of CD45, CD14, CD15, TLR2, and MACSQuant Running Buffer to the “FMO –TLR4” tube
- Incubate all of the tubes in the dark at 4°C for 10mins
- Following incubation, add 1ml of MACSQuant Running Buffer to each tube

Running the sample

- Open pre-determined Small Steps instrument bank settings, Small Steps analysis template and Small Steps experiment settings under File→Open
- Run 50ul of the “Full Stain” tube through the cytometer, use this to adjust the trigger to exclude most of the CD45- debris

- Add 2ul of Propidium Iodide Solution to the “Full Stain” tube and run 50ul through the cytometer again
- Repeat the above step for the 2 FMO control tubes

End of day

Put 500ul of 1% sodium hypochlorite in a 12x75mm round bottom tube and press the clean button on the flow cytometer

- Right click the water droplet button in the bottom left and select “Clean”
- Once the cytometer has finished cleaning click on the power button in the top right corner of the screen and select “Instrument off” from the pop-up menu

A.6 Whole blood culture technique.

Assay principle
A whole blood culture is technique that is used to measure how blood cells react to different stimuli. Specifically in this case we measured the functional ability of the immune cells within whole blood. This was done by measuring the cytokines released in response to stimulation with two molecules, LPS (a TLR4 agonist) and PAM-CSK4 (a TLR2 agonist).

Materials needed
- 15ml conical tubes, pipettes, pipette tips, 48 well plate.
- DMEM F0 5 mM glucose serum free with 1x pen/strep.
- Blood sample collected in 1 x 4 ml sodium heparin tube and kept at room temperature.
- LPS (Sigma-Aldrich) 1mg/mL made up to 1 pg/ml, 10 pg/ml and 100 pg/ml through dilituion with serum free media (F0).
- PAM-CSK4 (EMC) 0.5 mg/ml made up to 10 ng/mL and 100 ng/mL through dilution with serum free media (F0).

Initial preparation
- Pre-warm a 15 ml conical tube containing 12.6 mL of DMEM F0 5 mM glucose (serum free) with 1X Pen/Strep in the 37°C incubator with caps loosened. (This will warm media and equilibrate the pH of the media)
- Mix sodium heparin tube by gentle inversion 3-4X before pipetting blood as it may have settled.
    - Fasting blood will be collected in a 4 ml sodium heparin (green top) tube and inverted 6X upon venipuncture to mix blood with anticoagulant. The tube will be transferred to ASC 288 at room temperature.
- Pipette 1400 ul blood (2 X 700 ul) into the pre-warmed 12.6 ml DMEM tube to obtain 10-fold diluted whole blood. Mix by gentle inversion 6-8X.

Plating blood
- Pipette 540 ul of 10-fold diluted blood into wells of a 24-well plate. Follow the pattern given below.

	1	2	3	4
A	**X**	**X**	**X**	**X**
B	**X**	**X**	**X**	**X**
C	**X**	**X**		
D				
E				
F				

Label all plates with
    - Subject ID (SSXX)
    - Time of plating
- Place on top shelf of the CO2 incubator. Incubate the plate at 37°C for 15 – 30 min.

Treating blood
Add treatment either 60ul of LPS or 60ul of PAM-ck4. The total volume in each well is 600 ul

Plate Treatment (Designed for a 48-well Plate)

	1	2	3	4
A	**LPS 1 pg/mL**	**LPS 1 pg/mL**	**PAM-CSK4 10 ng/mL**	**PAM-CSK4 10 ng/mL**
B	**LPS 10 pg/mL**	**LPS 10 pg/mL**	**PAM-CSK4 100 ng/mL**	**PAM-CSK4 100 ng/mL**
C	**LPS 100 pg/mL**	**LPS 100 pg/mL**		
D				
E				
F				

Replace on top shelf of the CO2 incubator. Incubate the plate at 37°C until the first collection of supernatants.

Collecting supernatants.
At 24 hours of incubation:
- Centrifuge the 48 well plate at 2000 X g for 20 min. Transfer all of the supernatant into 1.5 ml labeled tubes. IMPORTANT: MAKE SURE THAT PLATES ARE ACCURATELY BALANCED, as this is the top speed for the rotor.
- Immediately store at -80°C.

A.7 ELISA for cytokines from whole blood cultures.

Assay principle
Cytokines produced from the whole blood culture are measured using ELISA which follows the same principle as the insulin ELISA (*appendix 1.2*). However for this ELISA wells needed to be coated with the binding antibody prior to adding the sample, unlike the insulin ELISA, in which the wells were pre coated. This ELISA was conducted without the use of the Chemwell and read on a bio rad plate reader.

Materials needed

- DuoSet ELISA kit – Human TNF-α, IL-10, IL-1β, IL-6.
    - Capture antibody
    - Detection antibody
    - Standard, using a 2 fold serial dilution to produce a 7 point standard curve.
    - Streptavidin-HRP (stop solution)
    - ELISA wash solution.

- Reagent diluent/blocking Solution. Dissolve 1% skim milk powder and 1% bovine serum albumin (BSA) in PBS (Ex. If making 50mL dissolve 0.5g skim milk powder and 0.5g BSA in 50mL PBS). Cover with parafilm and store at 4°C for up to 1 week.
- PBS (phosphate buffered saline) – Dissolve 1 tablet PBS (on shelf) for every 100ml dH_20. Store at room temperature in airtight bottle.

DAY 1
Preparation:

- Dilute capture antibody to working concentration in PBS.
- Coat plates with capture antibody, 100µL/ Cover plate with parafilm and incubate at room temperature overnight.

DAY 2

- Wash plate 3X with 400µl wash buffer.
- Using suction system, remove liquid from one or two wells and add 100µL of the corresponding samples.

<u>Block non-specific binding sites</u>:

- Block plates with 300µl reagent diluent.
- Incubate plate at room temperature for 1 hour.
  Wash plate 3X with 400µl wash buffer.

- Plate 100µL/well each sample and standard. Cover plate with parafilm and incubate at 2 hour.
- Wash plate 3X with 400µl wash buffer.
- To each well, add 100µL detection antibody.
- Incubate plate for 2 hour at room temperature.

- Wash plate 3X with 400µl wash buffer.
- Add 100µl of substrate solution to each well.
- Incubate for 20 min away from light.
- Add 50µl stop solution (streptavidin-HRP) to each well – tap to mix gently.

Measure Absorbance: using plate reader with a 450nm filter and 540nm correction filter.

Data analysis for ELISA

- Create an absorbance curve from standards and absorbance and check.
- Calculate the mean values for absorbances (A) for each sample.

A.8 Basal cytokine protocol for MAGPIX.

Assay principle

Multiplex immunoassay assays function with a similar principle as an ELISA, with some small differences. Multiplex immunoassays allow for the measurement of multiple analytes at once.

Magnetic beads (microspheres) with two colour coded fluorescent dyes each have a coating of a specific capture antibody. Once the specific analytes to be measured have been captured by the bead a detection antibody is added to the sample. Streptavidin PE is added to the wells, as this is the reporter molecule to excite the fluorescent dye on the bead.

Once within the MAGPIX the sample is brought up and placed in front of magnetic which holds the magnetic breads in place. Red and green LEDS are shined upon the beads and an image of the fluorescent beads is captured. Depending on the fluorescent pattern detected the analyte type and concentration is determined.

The multiplex immunoassay kit used for this assay was a custom 7 cytokine panel which measured protocol IL-6, IL-8, IL-1ra, TNF-α, MCP-1, IL-10, and IL-1β.

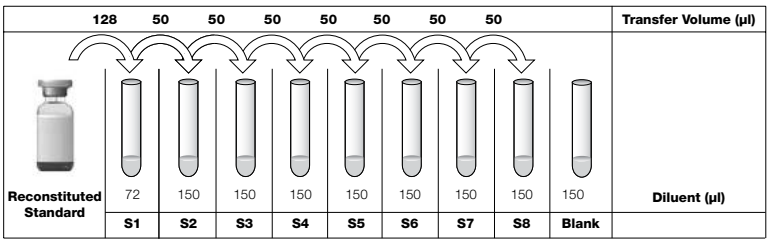
Materials needed

- MAGPIX reagent and bead kit – Bio-Plex Pro #171-304070M
- MAGPIX machine
- Pipette and pipette tips
- Plasma samples

Day one: (all measurement are made for a full 96 well plate)

Initial preparation

- Plan the plate layout
- Bring assay buffer, wash buffer and sample diluent to room temp, keep the other items on ice.
- Keep 10x coupled beads on ice – detection Abs can stay in fridge.
- Thaw frozen samples – once thawed spin at 10000 g for 10 min at 4 °C. Prepare samples, diluting them with a 1:1 ratio to make up to 50 µl per well.
    - If running singlets = 27 µl sample and 27 µl sample diluent.
    - If running duplicates = 55 µl and 55 µl sample diluent.
- Reconstitute a single vial of standards in 500 l of standard diluent. Vortex for 5 seconds and incubate on ice for 30 minutes.
- Prepare 4-fold standard dilution, as per instructions below – pipetting 50ul from each previous standard into the subsequent standard tube. Vortex for 5 seconds between each transfer. Diluent used is the standard diluent.



- Prepare the beads. Vortex for 30 seconds each before pipetting. Pipette 575 ul of each 10X beads cytokine into 1728 ul Assay buffer to make up 5760 ul in total. For the beads pipette 3 x 192 ul with a p200 tip. Protect from light.

Running the Assay

- Vortex the diluted (1X) beads for 20 seconds and pipette 50 ul into each well. Mix within trough with pipette between each column. This step should be done within 2 minutes to prevent bead clumping.
- Wash the plate two times with 100 ul of wash buffer.
  - Put plate on magnetic washer to bring beads to bottom.
  - Release from magnetic and add wash buffer, hold for up to 60 seconds.
  - Put plate back on magnetic washer and dump. Lightly tap on to paper towel.
- Vortex samples and standard and pipette 50 ul into each well.
- Cover with sealing tape and tinfoil. Incubate on shaker at 850 rpm in fridge overnight.

Day two

Initial preparation

- Turn on MAGPIX to warm up for 30 minutes.
- Bring assay buffer, wash buffer and sample diluent to room temp, keep the other items on ice.
- Prepare detection antibody. Vortex detection Ab before pipetting. Dilute from 10X to 1X as follows: Pipette 300 µl of each cytokine detection Ab into 900 µl of detection Ab diluent to make 3000 µl total.

Running the assay

- Remove shaker and plate from the fridge.
- Wash plate three times with 100 µl of wash buffer each time. Follow the steps from step 2 on day one.
- Vortex the 1X detection Ab and pipette 25 µl into each well.
- Cover with sealing tape and tinfoil and incubate on shaker on bench top at 850 rpm for 1 hour.

During this time calibrate and verify MAGPIX. Set up xPonent software protocol, selecting 7 panel kit.

- With 10 minutes left in the 1 hour incubation prepare the SA-PE. Vortex 100X SA-PE before pipetting. Dilute from 100X to 1X as follows: Pipette 60 µl of 100X SA-PE into 5940µl of assay buffer to make up 6000 µl total volume. Protect from light.
- Once the hour incubation is up wash three times with 100 µl of wash buffer, following the same steps as outlined on the first wash of day one.
- Vortex the 1X diluted SA-PE and pipette 50 µl into each well.
- Cover with sealing tape and tinfoil and incubate on shaker on bench top at 850 rpm for 10 minutes.
- Wash three times with 100 µl of wash buffer following the same steps as outlined on the first wash of day one.
- Pipette 125 µl of assay buffer into each well.
- Cover plate with sealing tape and shake on bench top at 850 rpm for 30 seconds.
- Remove sealing tape and read.

Once the read is complete, verify the accuracy of each standard curve for each cytokine and optimize each curve.

  - Download results for each cytokine to excel.

End of day procedure

Run the shut down procedure on the MAGPIX, selecting system shutdown from the auto maintenance menu.

- Eject the tray
- Fill reservoir 1 with MilliQ water, reservoir 3 with 20% bleach solution
- Retract the tray and run.

A.9 Estimated external work output for training.

Table 5: Estimated external work output (Kj) for HIIT and MICT when completed on a cycle ergometer and total duration of exercise (min) for each session during the two week intervention.

	Works units (Kj)		Total work out time (min)	
Session	HIIT	MICT	HIIT	MICT
1	650	655	14	24
2	810	817.5	16	29
3	930	947.5	18	33
4	930	947.5	18	33
5	1050	1045	20	36
6	1170	1175	22	40
7	1170	1175	22	40
8	1290	1272.5	24	43
9	1290	1272.5	24	43
10	1410	1500	26	50

HIIT = warmup and cooldown = 3min @ 35% Wpeak for all workouts
MICT = warmup and cooldown = 5min @ 20% Wpeak for all workouts

Appendix B: Supplementary data

B.1 Changes in systolic blood pressure

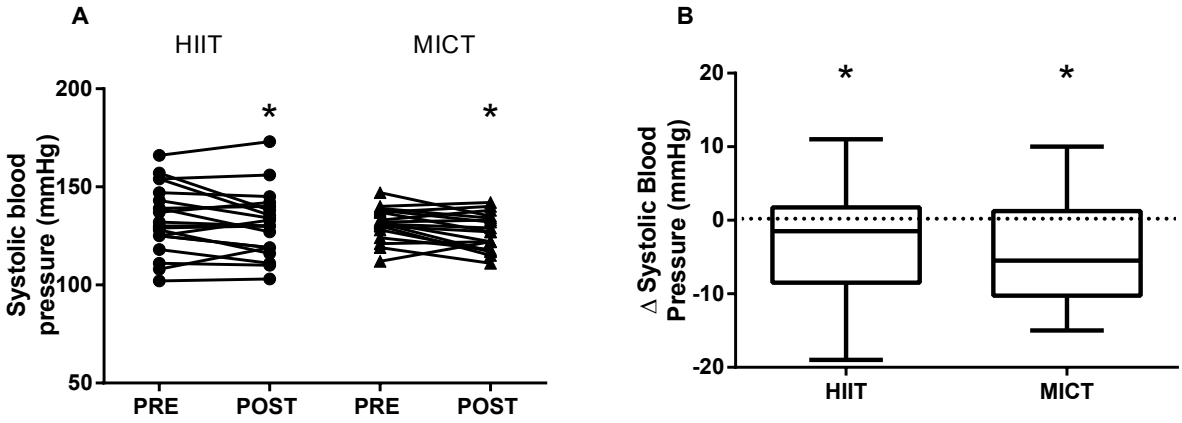


Figure 8: Two weeks of both HIIT (n = 20) and MICT (n = 18) decrease systolic blood pressure. Individual changes are shown by lines in (**A**) and change from pre-training is shown in the boxplot in (**B**). Main effect of time was significant (P<0.05). *significant increase from Pre-training within group (P<0.05).

B.2 Changes in diastolic blood pressure

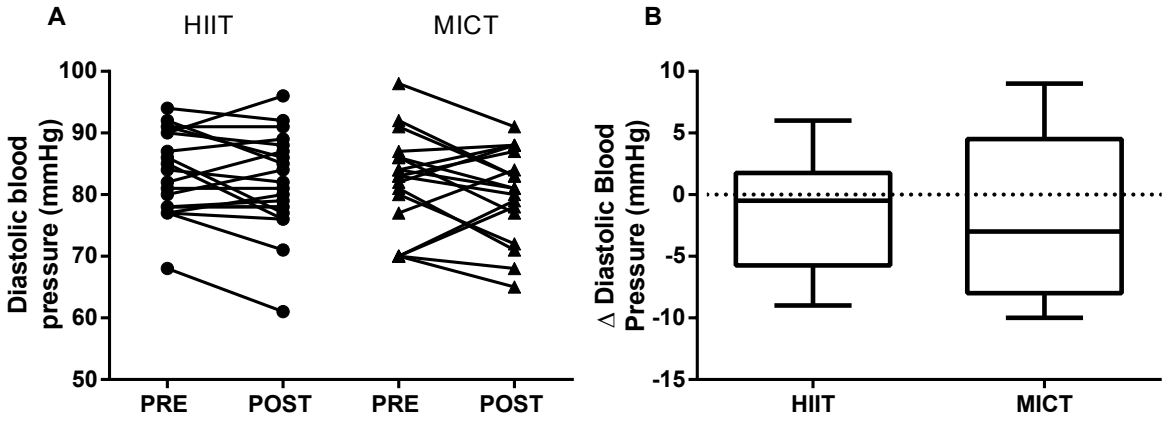


Figure 9: No change in diastolic blood pressure after two weeks of both HIIT (n = 20) and MICT (n = 18). Individual changes are shown by lines in (**A**) and change from pre-training is shown in the boxplot in (**B**). No main effect of time, group or interaction.

B.3 Changes in waist circumference

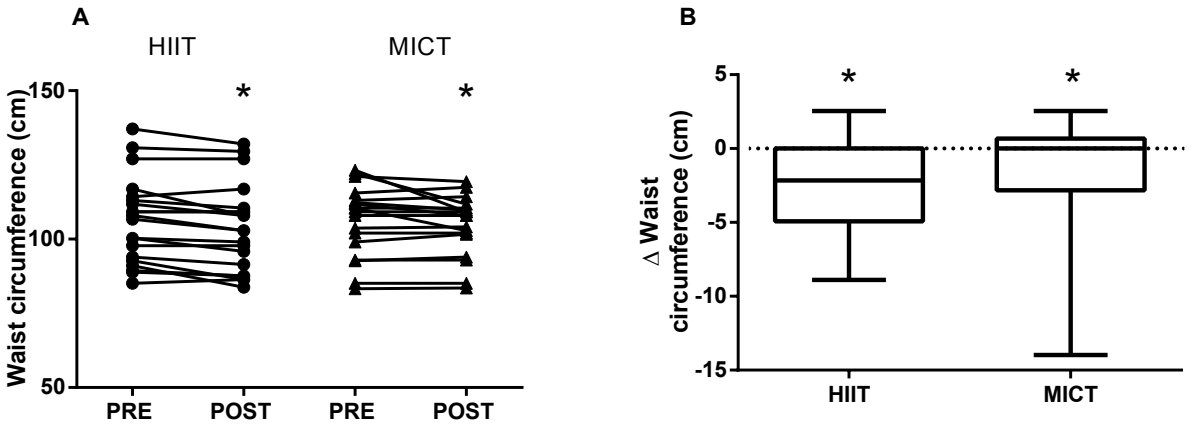


Figure 10: Two weeks of both HIIT (n = 20) and MICT (n = 18) decrease waist circumference. Individual changes are shown by lines in (**A**) and change from pre-training is shown in the boxplot in (**B**). Main effect of time was significant (P<0.05). *significant increase from Pre-training within group (P<0.05).

B.4 Changes in immune cell numbers

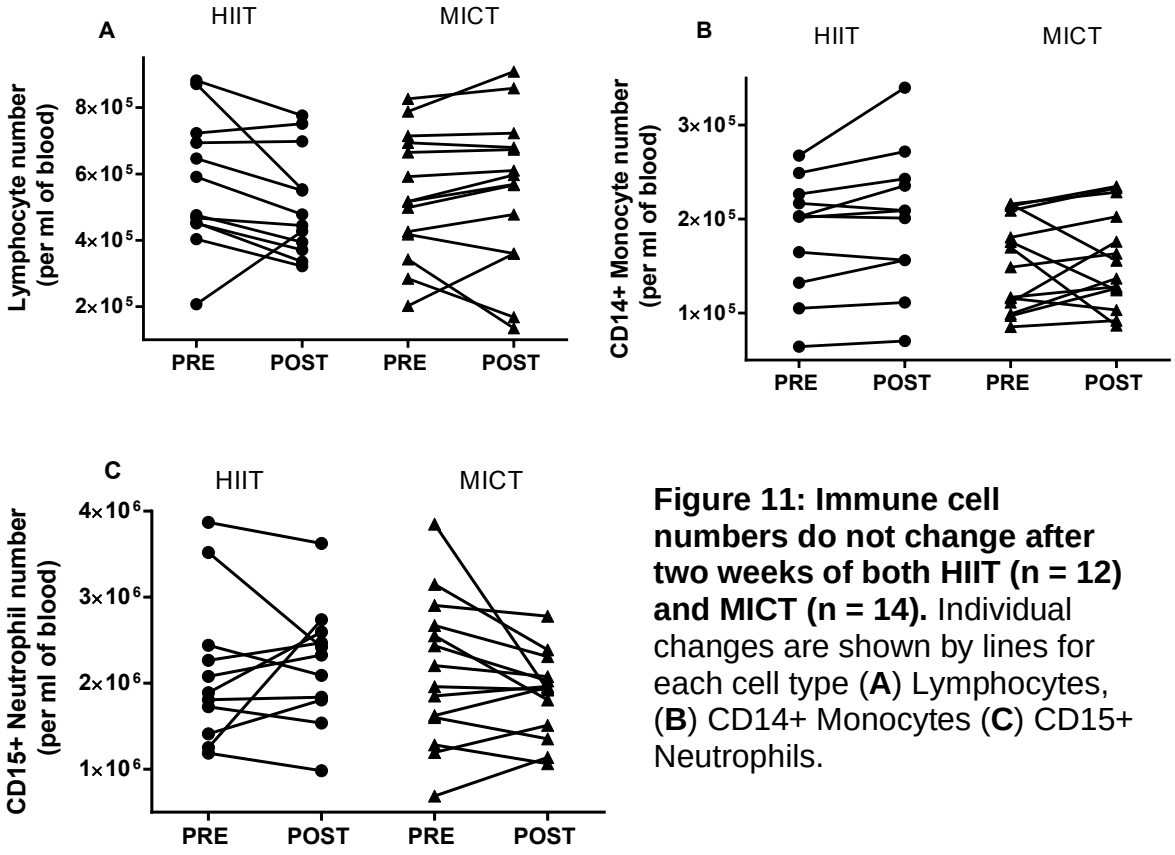


Figure 11: Immune cell numbers do not change after two weeks of both HIIT (n = 12) and MICT (n = 14). Individual changes are shown by lines for each cell type (**A**) Lymphocytes, (**B**) CD14+ Monocytes (**C**) CD15+ Neutrophils.

B.5 Changes in TLR2 on monocytes and neutrophils

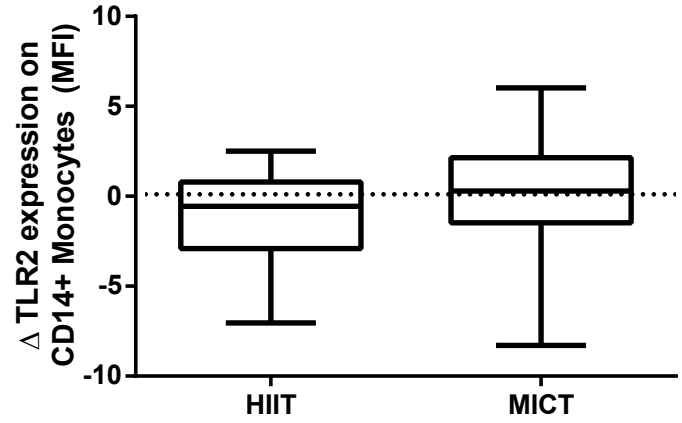


Figure 12: TLR2 expression on CD14+ Monocytes does not change after of either HIIT (n = 11) and MICT (n = 16). Mean change from pre-training is shown as a boxplot.

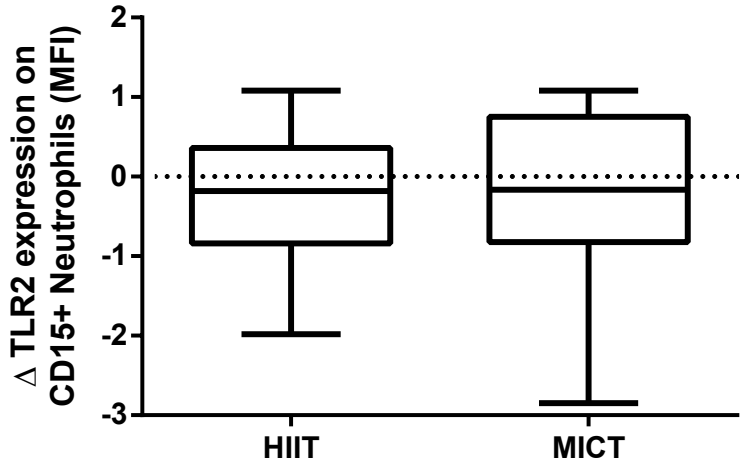


Figure 13: TLR2 expression on CD15+ Neutrophils does not change after of either HIIT (n = 12) and MICT (n = 17). Mean change from pre-training to post training is shown as a boxplot.

B.6 Correlation between change in TLR and plasma glucose

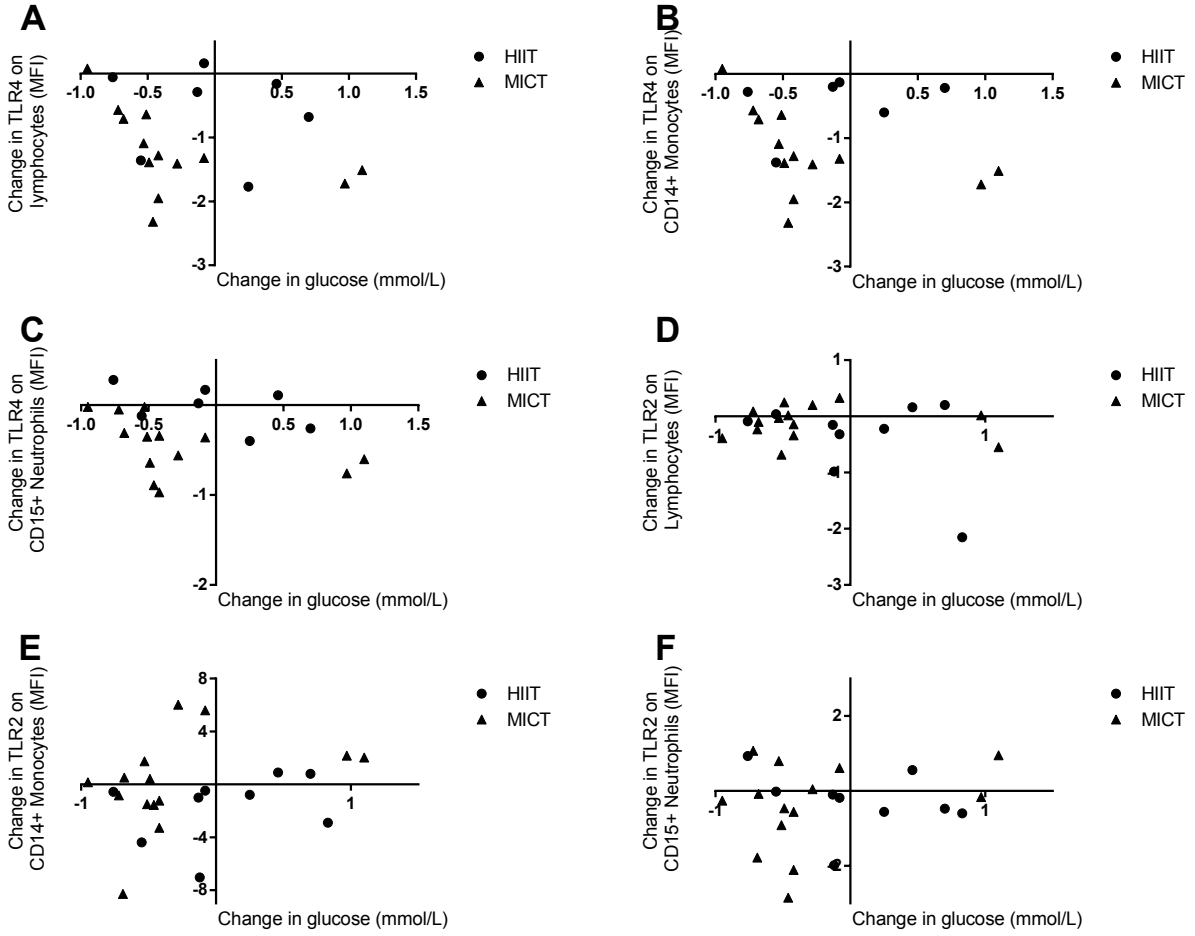


Figure 14: Changes in TLR 2 and 4 are not significantly correlated to change in fasting plasma glucose. No significant correlation between change in fasting blood glucose and TLR4 on lymphocytes (A, HIIT n = 7, MICT n = 13), CD14+ monocytes (B, HIIT n = 7, MICT n = 13), CD15+ neutrophils (C, HIIT n = 7, MICT n = 13) and between TLR2 on lymphocytes (D, HIIT n = 9, MICT n = 14), CD14+ monocytes (E, HIIT n = 9, MICT n = 14), CD15+ neutrophils (F, HIIT n = 9, MICT n = 14).

B.7 Correlation between change in TLR and body mass

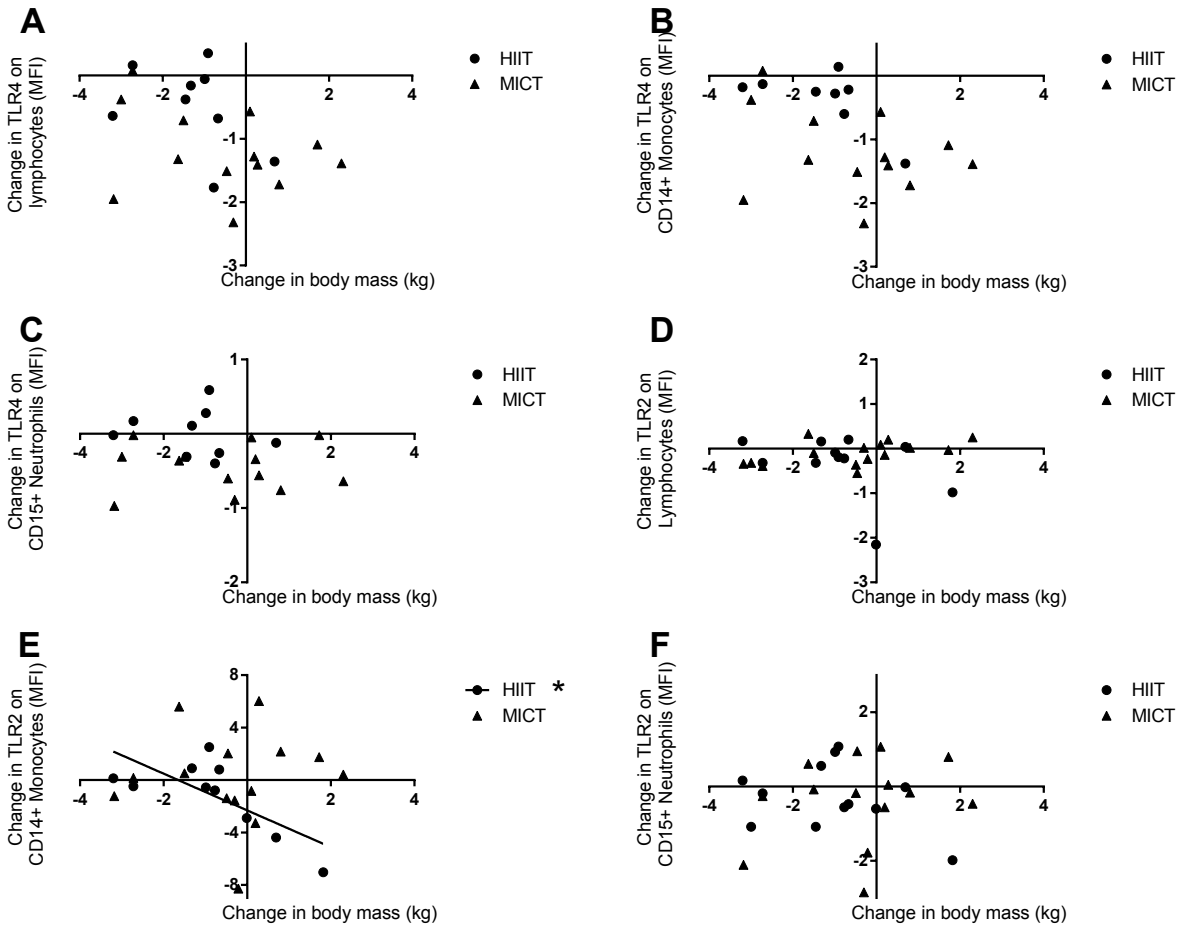


Figure 15: Changes in TLR 2 and 4 are not significantly correlated to change in body mass, with exception of TLR2 on monocytes. No significant correlation between change in fasting blood glucose and TLR4 on lymphocytes (A HIIT n = 9, MICT n = 14), CD14+ monocytes (B, HIIT n = 8, MICT n = 13), CD15+ neutrophils (C, HIIT n = 9, MICT n = 13) and between TLR2 on lymphocytes (D, HIIT n = 11, MICT n = 15) and CD15+ neutrophils (F, HIIT n = 11, MICT n = 15). Changes in TLR2 expression on CD14+ monocytes was negatively correlated to change in body mass (E, P = 0.01, HIIT n = 11, MICT n = 15).