# Are Myokines Linked to Metabolic Improvements with Increased Physical Activity in Subjects with Type 2 Diabetes: how Acute and Chronic Exercise Regulate Myokine Secretion in this Population.

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“eating alone will not keep a man well, he must also take exercise”

Hippocrates (460–370 BCE)

# Abstract

Skeletal muscle is the main site of post-prandial glucose uptake and in patients with type 2 diabetes (T2D), it is affected with metabolic dysfunctions. Muscle cells secrete peptides called myokines that positively modulate muscle metabolism. In patients with T2D, myokine secretion is altered. As physical activity is an efficient method for the management of T2D that can also regulate myokine secretion, regular practice of exercise could be beneficial to patients with T2D in restoring myokine secretion. To study this potential relationship, we explored the regulation of myokines in humans with or without T2D in response to acute and chronic exercise.

We first compared peripheral myokines in subjects with or without obesity in response to an exercise bout, and found that plasma FGF21, interleukin (IL)-6, IL-8, IL-15, and IL-18 were regulated by acute exercise, while obesity was associated with elevated plasma IL-13, but lower IL-8 and FGF21. Chronic inflammation is a common driver of both coronary artery disease (CAD) and T2D. The comparison of peripheral cytokines (candidate myokines) in subjects with CAD with or without T2D in response to interventions consisting of either high intensity interval training (HIIT) or moderate intensity continuous training (MICT) showed that the co-morbidity was associated with increased plasma IL-8 and training further reduced plasma FGF21 and IL-6 in this group. No differences were found between HIIT and MICT on cytokine secretion. Finally, we compared the effect of chronic exercise on peripheral and local myokine secretion in healthy subjects and patients with T2D with or without resistance to exercise training and found no differences as a function of T2D status or response to exercise, with the exception of modest differences in expression patterns for certain myokines (IL-1β, IL-8, IL-10, and IL-15).

This research serves as comparative reference of how autocrine/paracrine and endocrine signaling by skeletal muscle is altered by exercise in patients with T2D. Most studies concerning myokines

and their role in modulating energy metabolism mostly focus on a single candidate at a time, the results of my doctoral research are novel in considering the myokine network without neglecting the potential interactions in action of these peptides.

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# Table of Contents

**Abstract** ... iii

**Acknowledgements** ... v

**List of Abbreviations** ... x

**List of Figures** ... xiii

**List of Tables** ... xv

**Chapter 1** ... 1

**Introduction** ... 2

Literature Review ... 5

Diabetes Mellitus ... 5

Current Context of Type 2 Diabetes in the World. ... 6

Obesity as a Risk Factor for Metabolic Diseases ... 8

Skeletal Muscle in T2D ... 10

Muscle as a Secretory Organ ... 11

Role of Myokines in the Development of Skeletal Muscle Insulin Resistance and Related Metabolic Defects in Type 2 Diabetes ... 14

An In Vitro Model of the Muscle *Interstitium* During Contraction ... 46

Markers of Muscle Metabolic Adaptations to Exercise ... 47

Other Factors of the Muscle Secretome ... 48

Different Types of Exercise for the Management of T2D ... 49

The Concept of Exercise Resistance ... 50

**Statement of Objectives** ... 52

**Chapter 2** ... 53

**Plasma Myokine Concentrations After Acute Exercise in Non-obese and Obese Sedentary Women** ... 54

Abstract ... 55

Introduction ... 56

Materials and Methods ... 58

Results ... 61

Discussion ... 62

Conflict of Interest and Ethics ... 70

Author Contributions ... 70

Funding ... 70

Acknowledgements ... 71

Data Availability Statement ........ 71
References ........ 71
Figure ........ 75
Table ........ 76
Supplementary Material ........ 77
**Chapter 3 ........ 79**
**Exercise Training Reduces Circulating Cytokines in Male Patients with Coronary Artery Disease and Type 2 Diabetes: a Pilot Study ........ 80**
Abstract ........ 82
1. Introduction ........ 83
2. Results ........ 86
3. Discussion ........ 89
4. Materials and Methods ........ 98
5. Conclusions ........ 103
Author Contributions ........ 104
Grants ........ 104
Acknowledgments ........ 104
Disclosures ........ 104
References ........ 105
Figures ........ 110
Appendix ........ 113
**Chapter 4 ........ 114**
**Myokine Secretion Following an Aerobic Exercise Intervention in Individuals with Type 2 Diabetes With or Without Exercise Resistance ........ 115**
1. Introduction ........ 116
2. Results ........ 118
3. Discussion ........ 127
4. Materials and Methods ........ 130
References ........ 133
**Chapter 5 ........ 139**
**General Discussion ........ 140**
Summary of Findings ........ 140
The Search for the Exercise Factor ........ 143
The Involvement of Myokines in Chronic Low-grade Inflammation ........ 145
Study Limitations ........ 147

Future Directions ........................................ 149
Conclusion ........................................ 151
**References ........................................ 152**
**Appendix I ........................................ 170**
**Appendix II ........................................ 173**
Permissions for reprint ........................................ 173

## List of Abbreviations

| | |
|---|---|
| **6MWT** | six-minute walk test |
| **ACC** | acetyl-CoA carboxylase |
| **ALT** | alanine transaminase |
| **AMPK** | amp-activated protein kinase |
| **ANGPTL4** | angiopoietin-like 4 |
| **AS160** | Akt substrate 160 |
| **AST** | aspartate aminotransferase |
| **ATP** | adenosine triphosphate |
| **AUC** | area under the curve |
| **BAIBA** | β−aminoisobutyric acid |
| **BDNF** | brain derived neurotrophic factor |
| **BMI** | body mass index |
| **CABG** | coronary artery bypass graft |
| **CAD** | coronary artery disease |
| **CaMKII** | calcium/calmodulin-dependent kinase II |
| **CRP** | c-reactive protein |
| **CV** | coefficient of variation |
| **DAG** | diacylglycerol |
| **DEXA** | dual energy X-ray absorptiometry |
| **DNA** | deoxyribonucleic acid |
| **DPP-4i** | dipeptidyl peptidase-4 inhibitors |
| **ECG** | electrocardiogram |
| **EDTA** | ethylenediaminetetraacetic acid |
| **ELISA** | enzyme-linked immunosorbent assay |
| **EPS** | electro-pulse stimulation |
| **EVs** | extracellular vesicles |
| **FAP** | fibroblast activator protein alpha |
| **FBS** | fetal bovine serum |

| | |
|---|---|
| **FGF21** | fibroblast growth factor 21 |
| **GGT** | gamma-glutamyltransferase |
| **GIP** | glucose-dependent insulinotropic polypeptide |
| **GIR** | glucose infusion rate |
| **GLP-1RA** | glucagon-like peptide-1 receptor agonists |
| **GLUT4** | glucose transporter 4 |
| **HbA1c** | glycated hemoglobin A1c |
| **HFD** | high fat diet |
| **HIIE** | high-intensity interval exercise |
| **HIIT** | high intensity interval training |
| **HOMA-IR** | homeostatic assessment of insulin resistance |
| **HRR** | heart rate reserve |
| **hSkMCs** | human primary skeletal muscle cells |
| **IL** | interleukin |
| **IMAT** | intra-myocellular adipose tissue |
| **IR** | insulin resistance |
| **IRS-1** | insulin receptor substrate 1 |
| **LIF** | leukaemia inhibitory factor |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **MICE** | moderate intensity continuous exercise |
| **MICT** | moderate continuous intensity training |
| **miRNA** | micro RNA |
| **mRNA** | messenger RNA |
| **mtDNA** | mitochondrial DNA |
| **OXPHOS** | mitochondrial oxidative capacity |
| **PCI** | percutaneous coronary intervention |
| **PCr** | phospho-creatine |
| **PGC1a** | proliferator-activated receptor gamma coactivator 1 alpha |
| **PKC** | protein kinase C |
| **PMAT** | perimuscular adipose tissue |
| **PPAR** | peroxisome proliferator-activated receptor |

| | |
|---|---|
| **RNA** | ribonucleic acid |
| **ROS** | reactive oxygen species |
| **SAT** | subcutaneous adipose tissue |
| **SD** | Standard deviation |
| **SEM** | standard error of the mean |
| **SGLT2i** | sodium-glucose cotransporter 2 inhibitors |
| **SPARC** | secreted protein acidic rich in cysteine |
| **T2D** | type 2 diabetes |
| **TNF-a** | tumor necrosis factor-a |
| **VAT** | visceral adipose tissue |
| **$VO_2$max** | maximal respiratory capacity |
| **WAT** | white adipose tissue |
| **WHO** | world health organization |

# List of Figures

## Chapter 1

*Figure 1. Regulation of myokine secretion by acute and chronic exercise.................................. 18*

*Figure 2. Hypothetical mechanism of the development of muscle metabolic defects during type 2 diabetes and the potential involvement of myokines in this process........................................... 20*

## Chapter 2

*Figure 1. Myokines in circulation after an acute bout of exercise in women with or without obesity. ........................................................................................................................................ 75*

*Supplementary figure 1. Cross-reactivity of IL-15 antibody to the complex IL-15/IL-15Rα. ..... 78*

## Chapter 3

*Figure 1. Circulating cytokine concentrations in participants with CAD with or without T2D in response to a HIIT- or MICT-based rehabilitation program. .................................................... 110*

*Figure 3. Circulating cytokine concentrations in participants with CAD with or without T2D in response to a HIIT- or MICT-based rehabilitation program. .................................................... 111*

## Chapter 4

*Figure 1. Variations in resting cytokine serum concentrations following the 10-week aerobic exercise intervention. ........................................................................................................ 120*

*Figure 2. Variations in myokine secretion in cell culture supernatant of human primary myotubes in response to the 10-week aerobic exercise intervention. ......................................................... 121*

*Figure 3. Correlation analysis between myokine secretion in cell culture supernatant of human primary myotubes and serum concentrations of cytokines from the participants...................... 122*

*Figure 4. Effect of EPS on the phosphorylation of proteins involved in the AMPK-pathway in human primary myotubes quantified by immunoblotting. ...................................................... 123*

*Figure 5. Effect of electrode exposure control condition of human primary myotubes collected pre- and post-intervention on myokine secretion............................................................................... 124*

*Figure 6. Effect of EPS treatment of human primary myotubes collected pre- and post-intervention on myokine secretion.......................................................................................................... 125*

*Figure 7. Effect of the 10-week aerobic exercise intervention on the mRNA expression of genes linked to inflammation in human primary myotubes as assessed by NanoString multiplex assay.* ........ *126*

## Chapter 5

*Figure S1. The effect of exposure to CM obtained from stimulation of healthy myotubes on glucose uptake in these myotubes.* ........ *170*

*Figure S2. Quantification of myokine secretion from myotubes of healthy participants and patients with T2D in response to contraction.* ........ *171*

*Figure S3. Effect of exposure of healthy myotubes to the CM of myotubes from patients with T2D on glucose uptake with or without insulin treatment.* ........ *172*

# List of Tables

## Chapter 1

*Table 1. Secretion of myokines is altered by muscle contraction, regular exercise and T2D.* ...... *37*

## Chapter 2

*Table 1. Participant characteristics of women having completed a single, acute bout of moderate intensity continuous exercise.* ...... *76*

## Chapter 3

*Table 1 .Characteristics of the study participants pre- and post-training intervention* ...... *109*

*Appendix 1. List of medications taken by the participants at baseline (pre-) and follow-up visits (post-intervention).* ...... *113*

## Chapter 4

*Table 1. Participant characteristics pre- and post- 10-week aerobic exercise intervention.* ...... *119*

## Chapter 5

*Supplementary Table 1. Summary of results in myokine variations across studies.* ...... *143*

# Chapter 1

# Introduction

The management of type 2 diabetes (T2D) using exercise-based interventions is a field of research in continuous development, as we have yet to fully understand what protocols are best for this patient population and what molecular signaling mechanisms drive positive metabolic outcomes. My doctoral research project was based on the study of skeletal muscle secretory function in the context of obesity and metabolic disease, specifically T2D, and how exercise modulates the secretion of small peptides called myokines.

Non-communicable diseases such as heart disease, stroke, cancer, diabetes and chronic lung disease are responsible for almost three quarters of deaths worldwide according to the World Health Organization (WHO). Amongst the five major risk factors common to these non-communicable diseases is physical inactivity. Lack of regular physical activity contributes to the development of obesity, and this condition is strongly linked to the development of metabolic diseases such as cardiovascular disease and T2D. However, not all individuals with obesity develop complications from their condition and some can be categorized as metabolically healthy. Hence the interest in characterizing the putative molecular mechanisms that differentiate a metabolically sound physiological environment in obesity to one that leads to co-morbidities such as T2D and cardiovascular disease.

During the progression of T2D, the manifestation of insulin resistance (IR) in skeletal muscle and the liver precedes hyperglycemia and the clinical diagnosis of T2D. Many hypotheses have been put forward as to what molecular mechanisms contribute to the development of IR, but no consensus has been reached yet in the literature. As skeletal muscle is the primary site of glucose uptake after a meal, and an important tissue regulating metabolic adaptations to physical activity,

we chose to study the signaling mechanisms occurring in skeletal muscle following exercise. Specifically, we studied the regulation of peptides secreted by muscle cells called myokines, as their secretion has been shown as intrinsically altered in the context of T2D. Numerous studies have also demonstrated an important role for myokines in adaptions to skeletal muscle energy metabolism following acute and chronic exercise, as their levels fluctuate.

Interventions promoting an increase in physical activity in patients with T2D can be an integral part of their treatment regimen, as they can independently improve whole-body glucose homeostasis. That being said, exercise interventions are in some cases inefficient due to various factors including adherence and metabolic predisposition of the individual. There remains a lack of understanding regarding the molecular mechanisms that result in successful long-term metabolic improvements with exercise in patients with T2D. Modifications in myokine signaling in muscle could partially explain the improvements in muscle metabolic improvements or lack thereof. Meanwhile, studies of myokines in the context of exercise training as it relates to metabolic improvements in patients with T2D are for the most part inconclusive and oftentimes contradictory. To further advance the research on the role of myokines in the development of T2D, my PhD project explored the effects of acute and chronic exercise on circulating myokine concentrations, as well as their local muscle expression and secretion following chronic exercise in people with or without obesity, T2D (that respond or not to the beneficial effects of exercise training) and coronary artery disease (CAD).

The results of this research were equivocal, as few regulatory trends were detected regarding myokine secretion in response to either acute exercise, or the three different exercise interventions across the participant populations. It is possible, however, that these results could be explained by

the short duration of the protocols employed to detect differences in myokine secretion. It is now generally accepted that an active lifestyle prevents the development of a plethora of chronic non-communicable diseases including T2D, but no link has yet been drawn with muscle myokine signaling and habitual levels of physical activity. Follow up studies on the molecular mechanisms regulating myokine secretion in inactive or active individuals should be undertaken to discern potential correlations between functional and impaired energy metabolism in skeletal muscle as a function of myokine secretion patterns.

## Literature Review

### Diabetes Mellitus

The first recorded use of the term Diabetes Mellitus to characterize a state of illness in which individuals experience symptoms of glucose clearance through their urine dates back to over two millennia ago. The first effective pharmacological treatment for diabetes was introduced in 1922, after Frederick Banting, Charles Best, James Collip and John Macleod first discovered and then purified insulin at the University of Toronto[1]. Prior to the Nobel prize-winning discovery of insulin, patients diagnosed with diabetes were constrained to a strict low-calorie and no-carbohydrates diet to manage their symptoms. This diet, reminiscent of a prolonged fasting state, left the patients with a low quality of life and poor treatment outcome. We recently celebrated 100 years of insulin treatment availability, yet the pharmacological treatment approaches for diabetes remain focused on managing the symptoms, rather than preventing onset altogether.

The most prevalent types of diabetes mellitus in the population are type 1 and type 2 diabetes. Where type 1 diabetes manifests as an autoimmune disease centered around the pancreas losing its insulin secretion capacity due to β cell dysfunction, T2D originates from the development of an insensitivity to insulin in peripheral tissues called IR. In the early stages of T2D development, pancreatic β cells can compensate for IR in peripheral tissues by augmenting insulin secretion, but as hyperglycemia persists and IR progresses, β cell mass and function decrease leading to impaired glucose tolerance[2]. Hyperinsulinemia, initially a compensatory mechanism to prevent hyperglycemia, contributes to β cell failure and the progression to overt T2D. As of recently, the scientific literature characterizing the phenotypes of T2D includes more distinctions between individual patterns and/or trends in the manifestation of this disease[3]. Indeed, what was once

thought to be a disease born from either genetic factors or environmental factors leading to a unified outcome is now approached with a broader personalized phenotyping of each individual to better understand the nature of their symptoms. In the same line of thought, while T2D was previously thought to be treatable with a single scale of pharmacotherapies or lifestyle interventions suited for any patient population, the avenues of treatments offered to patients diagnosed with T2D now require more thorough adaptation and consideration.

**Current Context of Type 2 Diabetes in the World.**

The socio-economic burden of T2D is fast increasing globally. It is projected that by 2030, around 438 million people will be affected by diabetes, of which 90% are cases of T2D[4]. Between 2009 and 2019, the global estimated direct expenditures of diabetes-related healthcare in adults with diabetes nearly doubled, reaching US$760 billion. Canada ranked ninth in the world amongst nations for the highest total diabetes-related health expenditure in 2019[5]. According to Diabetes Canada, nearly one third of Canadians are affected with T2D or prediabetes and over half of these people cannot afford the prescribed treatment to manage their symptoms. Therefore, it seems essential to change our perspective and/or approach for the treatment and prevention of T2D, not only to reduce the costs associated with this epidemic, but also to improve the quality of life of a great number of Canadians.

An important clinical marker of whole-body glucose homeostasis is the percentage of glycated hemoglobin A1c (HbA1c), which serves to estimate glycemic control over the past two to three months. HbA1c is used as both a diagnostic tool to detect the onset of T2D, and as a reference for the choice of treatment(s) to avoid micro- and macrovascular complications due to chronic hyperglycemia. The clinical treatment guidelines vary to a certain extent, but it is generally accepted that a target HbA1c between 6.5-8% for individuals diagnosed with T2D can help lower

their risk of developing complications[6]. The first glucose-lowering medication prescribed to patients with T2D is more often than not metformin, as it is generally efficient, tolerable and low cost. Metformin treatment helps lower glycemia by downregulating hepatic gluconeogenesis through inhibition of glycerol-3-phosphate dehydrogenase, which increases the redox state, subsequently downregulating glucose production in the liver using lactate and glycerol as substrates[7]. As a secondary effect, metformin treatment also improves muscle insulin sensitivity, although the mechanism(s) by which this occurs remain to be completely elucidated. That said, secondary lines of treatment are commonly required to achieve and/or maintain an HbA1c below threshold. The second-line drug options listed by Diabetes Canada include: dipeptidyl peptidase-4 inhibitors (DPP-4i), glucagon-like peptide-1 receptor agonists (GLP-1RA), sodium-glucose cotransporter 2 inhibitors (SGLT2i), other insulin secretagogues (meglitinides and sulfonylureas), thiazolidinediones, and finally insulin. DPP-4i reduce fasting and post-prandial glycemia by increasing the half-life of endogenous incretins GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), thereby promoting insulin secretion (peripheral tissue glucose uptake) and inhibiting glucagon secretion (reduced hepatic glucose production)[8]. GLP-1RA, as their name suggests, activate endogenous GLP-1R signaling in the pancreas, heart, liver, brain and gastrointestinal tract, resulting in increased insulin secretion, lower inflammation, reduced glucagon secretion, increased perception of satiety and reduced gastric emptying and peristaltic movement[9]. SGLT2i contribute to lowering blood glucose levels by partially inhibiting glucose reabsorption in the renal proximal convoluted tubules, therefore increasing urinary excretion of glucose[10]. The mode of action of insulin secretagogues is to promote insulin secretion by the pancreas. For example, sulfonylureas induce exocytosis of insulin from pancreatic β cells by preventing the inflow of potassium ions ($K^+$) in the cells, inducing a depolarization of the cell

membrane, causing an inflow of calcium ions ($Ca^{2+}$) that causes contraction of the actomyosin filaments which releases insulin in the extracellular space[11].

T2D is increasingly considered as a heterogeneous metabolic disease, as both environmental and genetic factors can greatly impact the manifestation of its symptoms (*e.g.* insulin resistance and insulin insufficiency)[12]. When establishing which treatment options are best suited for a designated patient, these metabolic differences are crucial, as choosing between different drugs or drug combinations with or without an additive lifestyle intervention may yield completely different results as a function of the phenotypic expression of T2D in this patient.

**Obesity as a Risk Factor for Metabolic Diseases**

Prospective studies and meta-analyses have highlighted the increased risk of cardiovascular disease and T2D in people living with obesity compared to individuals with a healthy body mass index (BMI). Obesity is defined by increased whole-body adiposity and similarly to T2D, it manifests in a plethora of metabolic phenotypes in humans. The metabolic state of individuals with obesity was found to be influenced by the qualities of the adipose tissue accumulated, rather than solely based in the quantity stored in the body. Indeed, subcutaneous adipose tissue (SAT) versus visceral adipose tissue (VAT) storage, accumulation of adipose tissue within the liver and skeletal muscle, as well as the characteristics of visceral adipose tissue (*i.e.* macrophage infiltration and inflammatory state) are all associated with poor metabolic outcome in obesity[13]. In the longitudinal portion of the Nurses' Health Study conducted in the United States, the majority of women with obesity originally metabolically healthy transitioned to metabolically unhealthy over time[14]. Within this population, incidence of T2D and hypertension were the most increased over time. Obesity is a common risk factor to T2D and CAD, two chronic non-communicable

cardiometabolic diseases for which inflammation is an important factor in disease onset and progression.

One of the main causes for obesity is a poor diet and the imbalance between energy consumption and expenditure (positive energy balance). Excess energy from food intake is stored in adipose tissue, which can expand in both adipocyte size and number. White adipose tissue (WAT), where the majority of lipids are stored in the body, is an important regulator of whole-body homeostasis. SAT represents the largest WAT depot in the body, and in individuals with metabolically unhealthy obesity, its storage capacity is impaired leading to increased lipid accumulation in ectopic tissues (*e.g.* skeletal muscle, liver and heart), as well as in VAT[15]. This phenomenon of abnormal lipid accumulation is called lipotoxicity, as excessive ectopic storage of lipids causes inflammation in the affected tissues and IR. High levels of circulating free fatty acids during a lipid infusion as a model mimicking the effects of a sustained positive energy balance inducing lipid overload is sufficient to induce IR in muscle[16]. Moreover, the role of WAT stretches beyond storage of energy sources in the body, as it serves as an important endocrine tissue. Adipocytes can secrete a number of peptides, hormones, and cytokines collectively called adipokines. In the context of obesity, hypertrophic adipocytes secrete pro-inflammatory cytokines (interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 that can directly interfere with WAT insulin sensitivity, while also promoting the recruitment of macrophages and T-cell within the tissue, further exacerbating inflammation[17,18]. Conversely, adipose tissue secretion of adiponectin is reduced in individuals with obesity[19]. This adipokine is linked to increased insulin sensitivity in muscle, thus decreased adiponectin levels could also contribute to lower muscle insulin sensitivity with obesity[20]. Since the liver is in great proximity to VAT, it is highly susceptible to localized inflammation from this WAT depot, which favors the development of IR in this tissue.

**Skeletal Muscle in T2D**

Since muscle is the main site of glucose uptake in humans, it is an important tissue in the pathogenesis of T2D[21]. Studies of first-degree relatives of individuals living with T2D have shown that IR in skeletal muscle, the liver and likely adipose tissue as well, precedes hyperinsulinemia in the development of this disease[22]. However, it remains unclear whether IR develops first in skeletal muscle, liver or WAT, in the event that any occurrence is primary to the others. Interestingly, a small minority of individuals manifesting IR develop overt T2D, suggesting that factors linked to β cell dysfunction are necessary for the progression of this metabolic dysfunction[23]. In patients with T2D, some of the earliest metabolic defects are skeletal muscle IR and mitochondrial dysfunction[24]. Although muscle metabolic defects in T2D have been described extensively in the literature, the mechanism(s) initiating their development are not completely elucidated. It is not known whether muscle IR occurs before mitochondrial dysfunction or vice versa[25], but these metabolic alterations have been shown to contribute to one another[26]. A diet rich in lipids (high fat diet, HFD) can lead to mitochondrial overload and incomplete fatty acid oxidation[27]. This incomplete β-oxidation results in the production of numerous lipid intermediates that can interfere with muscle insulin signaling, resulting in IR[28-30]. For example, diacylglycerol (DAG) interferes with insulin signaling through insulin receptor substrate 1 (IRS-1), while ceramides affect Akt phosphorylation, both resulting in reduced downstream signaling and IR[29,31]. This underlines how behavioral factors, in this case dietary habits, can contribute to the risk of developing T2D. Sedentary behavior also increases considerably the risk of developing T2D, even after correcting for level of physical activity[32,33]. This suggests that time spent sitting (e.g. at work or watching television) independently of level of physical activity (any bodily movement produced by muscles that require energy expenditure, as defined by the WHO) influences T2D incidence. Bed-rest

studies have also shown that physical inactivity alone was sufficient to induce skeletal muscle IR and mitochondrial dysfunction in healthy subjects[34-36]. On the contrary, chronic exercise can increase skeletal muscle oxidative function and mitochondrial biogenesis, which results in improved whole-body insulin sensitivity through muscle metabolic adaptations[37]. Lifestyle modifications including regular exercise even proved to be more efficient for the management of T2D than metformin treatment alone[38].

**Muscle as a Secretory Organ**

Skeletal muscle secretes metabolites, microRNAs, extracellular vesicles (EVs) and peptides that mediate signal transduction locally, but can also be released in the circulation to act on other tissues[39-41]. The two first peptides that were described in the muscle secretome are myostatin[42] and interleukin (IL)-6[43,44]. As other peptides were identified as secreted by muscle specifically and many of them were also cytokines, Pedersen et al. proposed to call them "myokines"[45]. During muscle contraction, myokine secretion is increased, which suggests they might be involved in the metabolic adaptations of exercise in skeletal muscle[46]. Acute and chronic exercise affect myokine secretion differently and the regulation of these peptides both in muscle and in the circulation is specific to each myokine[46-50]. Regular exercise, for example an endurance training intervention, upregulates the resting circulating or muscle levels of myokines such as IL-10, fibroblast growth factor (FGF)21, irisin, leukemia inhibitory factor (LIF), and apelin, while it downregulates IL-6, myostatin, myonectin and musclin in humans and different animal models[51-58]. An acute bout of exercise, on the other hand, can regulate the release in plasma of IL-6, IL-15, FGF21, irisin and angiopoietin-like-4 (ANGPTL4). Some myokines, namely IL-6[59-63], IL-10[64,65], IL-13[66,67], IL-15[68,69], IL-18[70], FGF21[71,72], brain derived neurotrophic factor (BDNF)[73-75], apelin[76,77], irisin[78-80], myostatin[81], myonectin[82], LIF[83], ANGPTL4[84] and secreted protein acidic rich in cysteine

(SPARC)[85] improve muscle metabolism through increased oxidative function, fatty acid metabolism, mitochondrial biogenesis, glucose uptake, glucose oxidation and insulin sensitivity. A detailed description of how myokines are regulated by acute and chronic exercise in different secretory media, and how they may alter skeletal muscle metabolism can be found in our published literature review found further in this dissertation.

However, in the context of muscle IR and T2D, myokine secretion is altered[86]. Many myokines that have a positive effect on skeletal muscle metabolism are chronically elevated in the plasma and cell culture supernatant of human primary muscle cells (hSkMCs) from patients with T2D. Some of these include IL-6[86,87], IL-15[86,88], IL-18[89], FGF21[90,91], irisin[92-94], myostatin[95-97], myonectin[82], LIF[98], ANGPTL4[99,100] and SPARC[101,102]. Other myokines are downregulated in the context of IR, namely, IL-13[66] and BDNF[103]. The mechanisms by which some myokines are upregulated in T2D are not fully understood, but the increase in circulating myokines can be partially explained by the status of chronic low-grade inflammation that accompanies obesity and T2D[104]. Since myokines are signaling peptides that can be produced by other tissues/cells, such as the liver, adipose tissue and immune cells, levels in circulation are not necessarily representative of what is secreted by skeletal muscle. Therefore, in some cases, the increase in myokine levels might not originate from muscle cells. That said, as mentioned previously, the secretion of myokines from hSkMCs and their expression in muscle have been shown to be altered in IR. Since myokines mainly mediate beneficial metabolic adaptions of muscle during contraction, it seems contradictory to observe an increase in their secretion during T2D. A hypothesis that has been suggested is that the secretion of certain myokines, like IL-6, might follow a pattern similar to reactive oxygen species (ROS) production. In this sense, an acute and transient increase in IL-6 results in positive outcomes for muscle cells during exercise, while a chronic low-level elevation

of the secretion of this myokine results in IR, much like ROS[105]. This hypothesis is supported by the study of hSkMCs derived from individuals with obesity with or without T2D treated with recombinant IL-6, in which downstream signaling was impaired following acute IL-6 treatment compared to hSkMCs derived from healthy subjects[106]. Moreover, expression of IL-6 receptor alpha was downregulated in muscle biopsies from participants with obesity with or without T2D, potentially explaining reduced sensitivity to this myokine. This manifestation of altered muscle signaling in patients with T2D has been brought forward as a feature called "myokine resistance"[107].

Following is the integral text of a published literature review I co-authored to serve as an exhaustive description of the links between candidate myokines and T2D through their observed metabolic effects on skeletal muscle and previously assessed peripheral and local concentration in the context of IR. This review article was published in 2019 in the journal Diabetes and Metabolism.

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**Role of Myokines in the Development of Skeletal Muscle Insulin Resistance and Related Metabolic Defects in Type 2 Diabetes**

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## Abstract

Due to its mass, skeletal muscle is the major site of glucose uptake and an important tissue in the development of type 2 diabetes (T2D). Muscles of patients with T2D are affected with insulin resistance and mitochondrial dysfunction, which results in impaired glucose and fatty acid metabolism. A well-established method of managing the muscle metabolic defects occurring in T2D is physical exercise. During exercise, muscles contract and secrete factors called myokines which can act in an autocrine/paracrine fashion to improve muscle energy metabolism. In patients with T2D, plasma levels as well as muscle levels (mRNA and protein) of some myokines are upregulated, while others are downregulated. The signalling pathways of certain myokines are also altered in skeletal muscle of patients with T2D. Taken together, these findings suggest that myokine secretion is an important factor contributing to the development of muscle metabolic defects during T2D. It is also of interest considering that lack of physical activity is closely linked to the occurrence of this disease. The causal relationships between sedentary behavior, factors secreted by skeletal muscle at rest and during contraction and the development of T2D remain to be elucidated. Many myokines shown to influence muscle energy metabolism still have not been

characterized in the context of T2D in skeletal muscle specifically. The purpose of this review is to highlight what is known and what remains to be determined regarding myokine secretion in patients with T2D to uncover potential therapeutic targets for the management of this disease.

**Keywords:** Type 2 diabetes; insulin sensitivity; fatty acid oxidation; glucose homeostasis; myokines; physical activity

**Abbreviations:** type 2 diabetes (T2D), insulin resistance (IR), human skeletal muscle cells (hSkMCs), tumor necrosis factor-α (TNF-α), interleukin (IL), white adipose tissue (WAT), electro-pulse stimulation (EPS), body mass index (BMI), high fat diet (HFD), fibroblast growth factor 21 (FGF21), leukaemia inhibitory factor (LIF), secreted protein acidic rich in cysteine (SPARC), β-aminoisobutyric acid (BAIBA), angiopoietin-like 4 (ANGPTL4), mitochondrial DNA (mtDNA), intra-myocellular adipose tissue (IMAT), perimuscular adipose tissue (PMAT)

## Introduction

According to the World Health Organization, 422 million people were affected with diabetes in 2014, which amounts to 8,5% of the adult population in the world. Type 2 diabetes (T2D) is the most widespread form of this disease and its principal risk factor is excess body weight. Since skeletal muscle is a major consumer of glucose in the body due to its important mass, it plays an important role in the development of T2D[21]. Skeletal muscle of patients with T2D have been shown to develop insulin resistance (IR), as well as mitochondrial dysfunction[24,26]. A high fat diet (HFD) can cause incomplete fatty acid oxidation in the mitochondria of skeletal muscle cells[27]. The lipid metabolites (e.g. fatty acyl CoAs, acylcarnitines, ceramides and diacylglycerol) resulting from this incomplete β-oxidation interfere with insulin signalling[27,28,30]. For example, diacylglycerol induces the phosphorylation of Ser/Thr of IRS-1 by Protein kinase C (PKC) β/δ, while ceramides interfere with Akt phosphorylation in human skeletal muscle, both resulting in inhibition of downstream signalling and IR[29,31].

Chronic low-level inflammation is a known feature of obesity and an important factor in the development of T2D. In the white adipose tissue (WAT) of subjects with obesity, macrophages accumulate and cause chronic inflammation that eventually leads to IR. As Hotamisligil reviewed in 2017, the cytokine tumor necrosis factor-α (TNF-α) is central to the link between inflammation, obesity and T2D, but other factors are also involved. The role of these factors including other cytokines, RNAs and metabolites are not fully understood yet due to the complexity of the interaction networks between these molecules and the hierarchy in their mechanisms of action.

The efficacy of physical activity for the management of T2D has been demonstrated many times[108]. A lifestyle intervention involving regular physical activity was found to be more effective in

reducing T2D incidence than Metformin treatment alone[38]. Regular exercise induces mitochondrial biogenesis and increased oxidative function in skeletal muscle, resulting in improved whole-body insulin sensitivity[37]. In contrast, physical inactivity is generally associated with higher risks of developing T2D[109]. Furthermore, sedentary behaviour such as watching television or sitting at work increased the risk of developing both obesity and T2D in individuals independently of their level of physical activity[32]. On the contrary, moderate exercise such as brisk walking for an hour daily was sufficient in reducing the risk of T2D by about 34%. In bed-rest studies, it was shown that as short as 5-7 days of inactivity in healthy volunteers resulted in increased IR[110,111]. This reduced insulin sensitivity was not related to a decreased insulin response of the liver, suggesting that sedentary behavior induced IR primarily in skeletal muscle[111]. It was also shown that bed-rest induced a reduction in muscle mitochondrial function[36]. Taken together, the literature suggests that muscle metabolic defects affecting patients with T2D could arise from their sedentary behavior.

Physical activity is recognized for the management of T2D, but also other diseases such as cancers, cardiovascular diseases, arthritis, depression and anxiety. How muscle contraction affects the function and physiology of distant organs is not clear, but one hypothesis is that these effects are mediated by the secretion of small peptides by contracting skeletal muscle. One of the first peptides identified to be secreted during muscle contraction was interleukin (IL)-6[43,44]. Other factors were then shown to originate from contracting muscle and in 2003, Pedersen et al. proposed that cytokines originating from skeletal muscle should be called myokines[45]. This group also described the role of IL-6 in regulating muscle energy metabolism and reducing chronic low-grade inflammation. Both chronic and acute exercise regulate myokine secretion (Fig. 1).

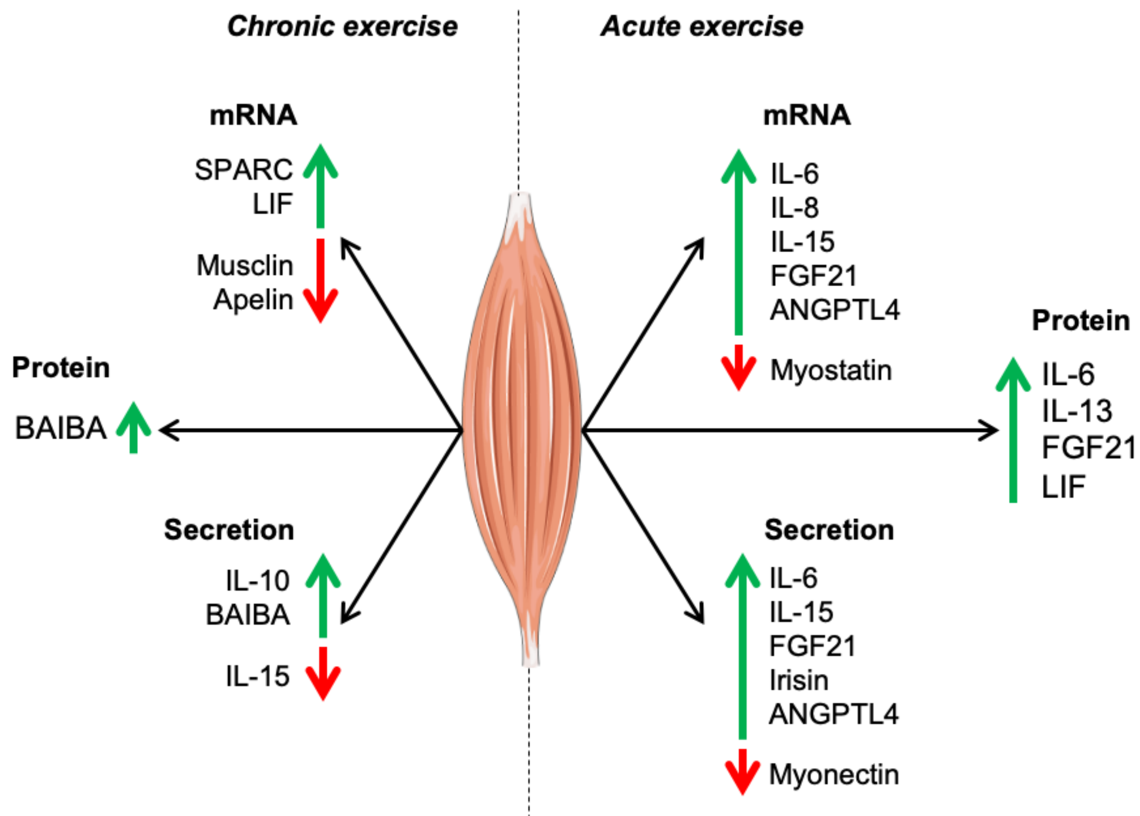


*Figure 1. Regulation of myokine secretion by acute and chronic exercise. Acute and chronic physical exercises result in different alterations of myokine expression at the mRNA or protein levels in skeletal muscle and of myokine secretion in circulation. Expression or secretion of most recognized myokines is increased by acute or chronic exercise, but some is decreased in response to exercise.*

Other factors than myokines are secreted during muscle contraction such as RNAs and metabolites to form the muscle secretome[41]. Myokines can improve muscle metabolism locally or be released in the circulation to act in an endocrine fashion to improve the function of other organs such as the liver, pancreatic b cells or adipose tissue[39,40]. Interestingly, myokines are secreted differently in patients with T2D. Indeed, the myokine profile of human primary skeletal muscle cells (hSkMCs) from patients with T2D is different from insulin sensitive hSkMCs[86]. Since the muscle secretome has only recently drawn attention in the context of metabolic diseases, few studies have been completed for the comparison of myokine secretion between individuals with IR/T2D and healthy subjects. Circulating levels of some myokines are downregulated in patients with T2D in

comparison to healthy subjects, while others are increased[112]. This suggests that myokine secretion and/or signaling is altered in people with T2D and it is possible that the lack of physical activity in people with T2D[109] results in these alterations. Since myokines are known to increase muscle insulin sensitivity and mitochondrial function, an alteration in myokine secretion in patients with T2D could potentially lead to skeletal muscle IR and mitochondrial dysfunction (Fig. 2). Therefore, the purpose of this review was to clarify the role of myokines potentially involved in the development of muscle IR and mitochondrial dysfunction. The focus of the review is on the autocrine/paracrine effect of myokines in altering energy metabolism of skeletal muscle cells in the context of T2D. A major challenge in the observation of autocrine/paracrine effects of myokines on muscle energy metabolism is the fact that circulating levels of these peptides are not reflective of their interstitial concentrations within skeletal muscle. During muscle contraction, certain myokines are released into the circulation, but others remain in the muscle interstitium to act locally. To better understand the effect of contraction-induced myokines on skeletal muscle metabolism, it is crucial to focus on the local levels of these peptides.

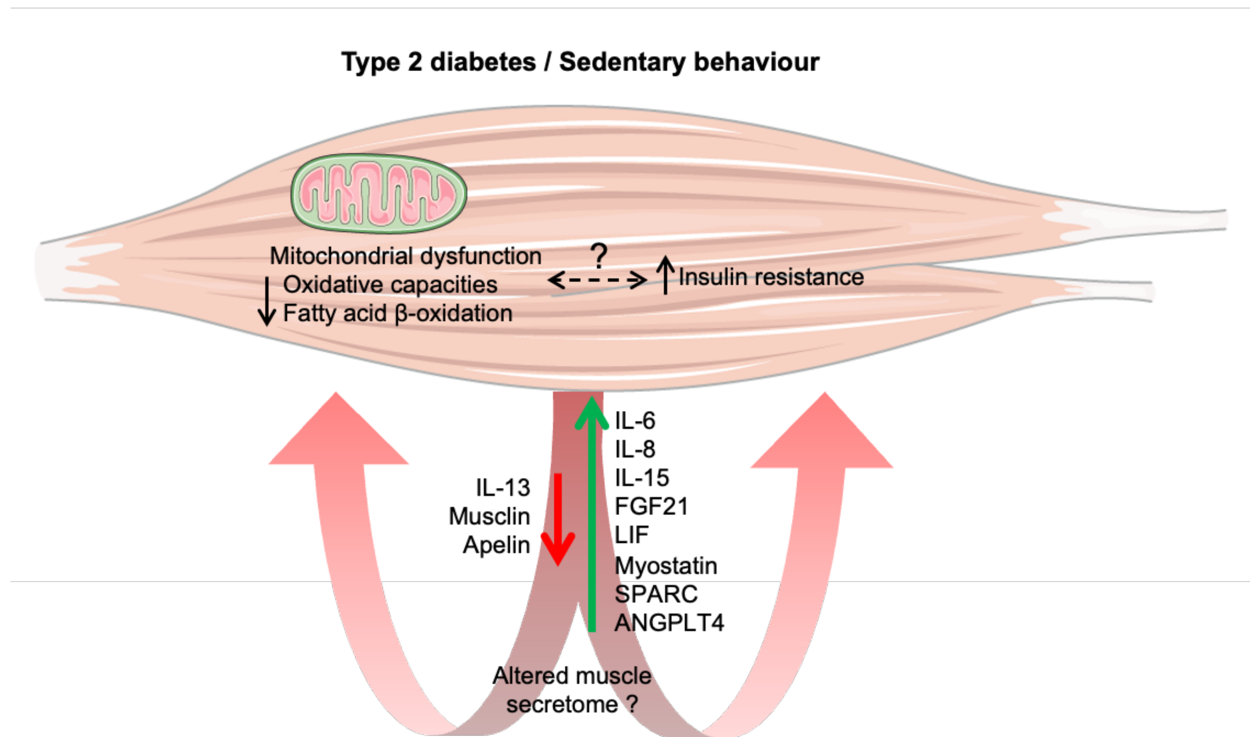


*Figure 2. Hypothetical mechanism of the development of muscle metabolic defects during type 2 diabetes and the potential involvement of myokines in this process. The muscle secretome (i.e. secretion of myokines, RNAs, mtDNA and metabolites) is altered in the context of type 2 diabetes and/or sedentary behavior. This altered muscle secretome can potentially lead to an altered mitochondrial function and/or insulin resistance development in skeletal muscle.*

**Interleukin 6**

Back in 2005, Petersen and Pedersen reviewed the role of IL-6 in the development of metabolic disease and proposed that this myokine could serve as a marker of T2D rather than a cause as it was previously believed[113]. IL-6 was the first identified myokine and is a particular cytokine since it can be produced by almost any cell type with the right stimulus[114] (i.e. TNF-α treatment induces IL-6 expression in fibroblasts)[115]. This particularity could explain why studies in the literature focusing on levels of IL-6 in IR conditions are contradictory and differ according to the tissue. As Pedersen and Febbraio described it, “the role of IL-6 in inflammation is context dependent”[116]. Systemic levels of IL-6 are elevated in the context of obesity and T2D and it has been shown that plasma IL-6 levels are correlated to whole-body adiposity[87]. This suggests that high systemic

levels of IL-6 are related to poor metabolic outcomes (i.e. development of IR, obesity, impaired fatty acid oxidation). That said, several studies have shown the inhibitory effect of IL-6 on TNF-α[113]. TNF-α is a main factor of chronic low-grade inflammation that has been identified as one cause for the development of IR[117]. IL-6 could then be involved in reducing chronic inflammation by inhibiting TNF-α production.

In skeletal muscle, IL-6 is acutely induced by contraction: circulating IL-6 levels can be increased up to 100-fold in response to an acute bout of exercise[44]. It has also been demonstrated that IL-6 ameliorates insulin-stimulated glucose disposal probably through its positive effect on muscle energy metabolism (increase in muscle glucose uptake, insulin sensitivity and fatty acid oxidation)[62]. The improved glucose conversion to glycogen, as well as the increase in fatty acid β-oxidation with acute and chronic treatment of muscle cells with supra-physiological concentrations of IL-6 is mediated by the AMPK pathway[61,62]. It was also shown that acute supra-physiological treatment with recombinant human IL-6 increased lipid oxidation in muscle without affecting WAT lipolysis, suggesting an autocrine/paracrine role of muscle-secreted IL-6 in increasing lipid metabolism[59]. That said, this finding does not exclude the possibility that chronic IL-6 exposure could eventually lead to increased fatty acid release from WAT to provide substrates for muscle β-oxidation, as the researchers highlighted. High levels of IL-6 in skeletal muscle seem to have a different effect depending on the exposure time. As demonstrated by Nieto-Vazquez et al., acute IL-6 treatment of hSkMCs increased insulin stimulated glucose uptake, while chronic treatment lead to impaired insulin signalling[63]. This feature of IL-6 signalling resembles ROS production within skeletal muscle, which is very acute during a bout of exercise and modulates energy metabolism positively, while in the context of T2D, its chronic elevation leads to the development of skeletal muscle IR[105]. An acute increase in IL-6 secretion during exercise could then be

beneficial for muscle energy metabolism, while a chronic elevation of IL-6 secretion in the context of T2D could lead to IR and mitochondrial dysfunction.

Muscle *IL-6* mRNA levels as well as circulating IL-6 has been measured in healthy individuals and patients with T2D in response to an acute bout of exercise[52]. No differences were found in the expression of *IL-6* mRNA after exercise between healthy and T2D muscle, but results suggest a trend towards a greater increase in muscle *IL-6* mRNA expression after exercise in patients with T2D. Also, their results showed no correlation between plasma IL-6 and mRNA in muscle, suggesting that other tissues contribute to circulating levels of IL-6. Increased circulating levels of IL-6 in the context of T2D and obesity could be explained by adipose tissue inflammation rather than the muscle secretion of this cytokine. As far as we know, IL-6 concentration in muscle interstitium specifically has not been compared between healthy subjects and patients with T2D. However, cultured hSkMCs from patients with T2D secrete more IL-6 at rest than myocytes from non-diabetic subjects[86], suggesting that increased circulating IL-6 in patients with T2D could be accountable to muscle secretion. Unfortunately, to our knowledge, IL-6 secretion after muscle contraction in the interstitial space or electro-pulse stimulation (EPS), which mimics muscle contraction *in vitro*, of hSkMCs has not been compared between insulin sensitive and IR individuals. On the other hand, Jiang et al. demonstrated that human hSkMCs obtained from participants with T2D respond differently to IL-6 treatment than healthy hSkMCs[107]. Indeed, IL-6 treatment did not increase glucose uptake in IR hSkMCs as much as in healthy hSkMCs, but the positive effect on lipid oxidation was similar in both conditions. This suggests that IL-6 signalling is partially altered in hSkMCs from patients with T2D. Their increased IL-6 secretion might be a compensation mechanism to counteract the alteration in muscle IL-6 signalling, similar to increased insulin secretion in the context of IR. Taken together, these studies suggest that muscle

from patients with T2D are “IL-6 resistant”. Further investigations would be required to test that hypothesis.

**Interleukin 8**

It has been reported in healthy subjects that *IL-8* mRNA is increased up to 10-fold in muscle after exercise and up to 2-fold in a model of myotube contraction[118]. Nielsen and Pedersen suggested that since *IL-8* mRNA is dramatically increased by exhaustive exercise (3h of treadmill running) as an inflammatory response and increased to a lesser extent in muscle after a bout of exercise of moderate intensity (1h of cycling), but circulating IL-8 is not significantly increased in these conditions, this myokine might act on muscle in an autocrine/paracrine manner[119]. In this sense, it was shown that EPS stimulation of hSkMCs induced an increase in IL-8 secretion, further confirming the secretion of IL-8 by muscle cells[120].

This cytokine has previously been associated with obesity and/or IR through positive correlations between circulating levels of IL-8 and BMI (body mass index), as well as HOMA-IR[121]. In hSkMCs affected with IR, IL-8 secretion was found to be increased significantly[122]. Similarly, IL-8 secretion was increased in hSkMCs and skeletal muscle tissue explants from patients with T2D in comparison to healthy subjects[86,123]. That said, circulating IL-8 levels were not different between the two subject groups, which contradicts the results of Zozulinska et al. who had found elevated levels of plasma IL-8 in patients with T2D compared to healthy subjects[124]. The role of IL-8 in the development of skeletal muscle IR remains unclear since most studies on this myokine in the context of T2D focused on circulating levels of this inflammatory factor, which do not seem to represent the level of the myokine in muscle interstitium.

**Interleukin 10**

IL-10 is a cytokine expressed in numerous tissues such as the heart, liver and adipose tissue, as well as skeletal muscle. In a model of IR in mice, Hong et al. demonstrated the protective effect of supra-physiological levels of IL-10, as well as muscle specific overexpression of IL-10 in the reduction of IR and inflammation in skeletal muscle[64]. Similarly, obese mice overexpressing muscle specific IL-10 were gaining weight under a HFD, but their muscle insulin signalling capacities and glucose metabolism were increased[65]. Since IL-10 acts in a positive way on skeletal muscle metabolism, it is of interest for the study of its role in the development of IR and mitochondrial dysfunction. Interestingly, it has been shown that low production capacity of circulating IL-10 in response to inflammation is associated with T2D, meaning that patients with T2D secrete IL-10 differently in response to an inflammatory stimulus[125]. In a recent study, patients with T2D underwent a 12-week high intensity interval training exercise program and plasma quantification of IL-10 revealed no change in levels of this myokine after an acute bout of exercise, but a significant increase at the end of the intervention in the resting state[53]. Unfortunately, to date, IL-10 secretion by skeletal muscle cells *per se* has not been detected in response to contraction *in vivo* or *in vitro*[86,120]. Since this myokine seems to induce positive muscle metabolic changes in an autocrine/paracrine manner, measuring the levels of IL-10 in muscle interstitial space in the context of T2D would be more informative than only quantifying its circulating levels.

**Interleukin 13**

IL-13 is a contraction-induced myokine that has been studied for its positive effect on energy metabolism[120]. Indeed, Darkhal et al. showed that the global overexpression of IL-13 in mice

reduced the inflammatory response and the development of IR caused by a HFD[67]. Interestingly, IL-13 levels in serum and secretion from hSkMCs were shown to be lower in patients with T2D, but not the muscle mRNA expression or the presence of IL-13 receptor (IL-13RA1)[66]. The authors suggested that this implied a post transcriptional regulation mechanism of IL-13 in which unknown T2D related factors were involved. hSkMCs from individuals with T2D did not show an equivalent increase in basal glucose uptake after exposition to IL-13 as obtained in healthy hSkMCs, although glucose oxidation was similar, suggesting a partially altered IL-13 signalling pathway in the context of T2D. Further exploration of the mechanisms regulating IL-13 secretion in the context of T2D could help better understand how this myokine modifies muscle energy metabolism and how the response to IL-13 signalling can be improved in IR muscle.

**Interleukin 15**

IL-15 was first described as expressed in skeletal muscle in 1995 by Quinn et al., where it was found to induce muscle fiber hypertrophy. A significant correlation between body composition markers (i.e. BMI, trunk fat and total body fat percentage) and circulating IL-15 levels has been shown in humans. Body composition correlated negatively with IL-15 in plasma, while T2D status did not[126]. However, a more recent study showed an increase in plasma IL-15 in patients with T2D and a downregulating effect of chronic exercise on basal circulating levels of IL-15 regardless of body composition[88]. An increase in resting IL-15 secretion by hSkMCs from T2D patients in comparison to healthy subjects was also found[86].

In mice, it was shown that IL-15 was necessary for the positive effect of exercise on muscle PPARδ pathway activation[68], suggesting IL-15 is at least in part involved in the positive effect of exercise on muscle oxidative capacities. In a mouse model, muscle *IL-15* mRNA content was found to be

reduced by HFD-induced obesity, while treadmill running increased muscle *IL-15* mRNA[127]. This contraction induced increase in muscle *IL-15* mRNA was also observed in humans after resistance exercise[128]. Contradictorily, no alteration in muscle *IL-15* mRNA was found after exercise in obese subjects with or without T2D, while plasma IL-15 levels increased[52]. These discrepancies in the literature regarding muscle and circulating levels of IL-15 in the context of T2D and/or in response to exercise might be due to the intricate role of the subunit α of the IL-15 receptor (IL-15Rα). The secreted form of IL-15Rα (sIL-15Rα) can be an agonist or an antagonist of IL-15 depending on the cell type. The methods used to measure circulating IL-15 in the above mentioned studies were not necessarily ascertained as specific to the free form of IL-15, which might be the active form of the myokine that targets muscle cells. The complexed form (IL-15/sIL-15Rα) might also have been detected in these studies (reviewed in[129]). Further observations focusing on the quantification of the free form of IL-15 and IL-15/sIL-15Rα in muscle interstitium, especially after muscle contraction, could help unveil the potential role of this myokine in T2D.

**Interleukin 18**

IL-18 is a cytokine expressed in various human tissues including skeletal muscle and is found mostly in type II glycolytic fibers[130]. Around fifteen years ago, Aso et al. demonstrated that systemic levels of IL-18 were significantly increased in the context of T2D[89]. More recently, IL-18 treatment was found to increase muscle lipid oxidation in IR mice through the AMPK pathway[70]. These findings suggest that high levels of IL-18 in the plasma might be related to a chronic inflammation status, while an acute increase of this myokine in muscle improves its metabolic functions. Nonetheless, to our knowledge, no studies have shown the effect of muscle contraction on the secretion and/or muscle mRNA expression of IL-18.

## Musclin

Musclin is a myokine known for its potential involvement in the development of IR[131]. A recent study showed that plasma levels of musclin were positively correlated to some indicators of T2D such as fasting plasma glucose and triglycerides, as well as whole-body IR[132]. This finding is supported by an earlier study from Liu et al., who showed that exposition of rat muscle cells to musclin reduced glucose uptake by inhibiting insulin signalling through Akt[133]. Another study showed an increase in muscle protein and mRNA levels of musclin in IR rats in comparison to controls[134]. The same group showed a reduction of the expression of musclin mRNA in muscle of IR rats after an exercise intervention[56]. Since this myokine seems to be, at least in part, involved in the development of IR, further studies on musclin levels in muscle after an acute bout of exercise, as well as a comparison in the exercise response between healthy and muscle cells from patients with T2D are needed. The secretion of musclin in response to muscle contraction has yet to be demonstrated.

## Fibroblast growth factor 21

Fibroblast growth factor 21 (FGF21) secretion from muscle of patients with T2D is increased[91]. It has been suggested that this myokine is involved in IR, which was supported by Lindegaard and colleagues who demonstrated that *FGF21* mRNA expression is increased in skeletal muscle of individuals with IR but not in their plasma[90]. Recently, it was shown that first-degree family history of T2D is not a predictor of elevated circulating FGF21 levels[135]. This suggests that the elevation of FGF21 in the plasma does not precede T2D, but it does not rule out elevated muscle *FGF21* mRNA as an early event in the development of this disease. Interestingly, Voigt et al. found that induction of FGF21 expression by mitochondrial stress reduced IR in a diabesity mouse model[72].

The positive effects of this myokine on metabolic disease were mediated by its stimulation of adiponectin secretion[71]. Adiponectin is produced by adipose tissue and is downregulated during obesity and T2D, causing a reduction in muscle fatty acid oxidation and insulin sensitivity[136]. Conversely, FGF21 was increased in the circulation, as well as at the mRNA and protein levels in muscle after an acute bout of exercise[137]. Others found that eccentric exercise had no effect on either muscle FGF21 content or release in the circulation, although they did detect an increase in fibroblast activator protein alpha (FAP), which negatively regulates FGF21 activity[138]. They did not detect FAP release from skeletal muscle, which indicates this mechanism of regulation might not be related to muscle contraction *per se*, but rather to other metabolic adaptations to exercise. Also, the authors mentioned that the eccentric exercise applied was submaximal, which is sufficient to induce IL-6 secretion by muscle, but could be insufficient for the release of FGF21 in the circulation since the stress component of more strenuous exercise is absent. In a recent study by Sabaratnam et al., an acute exercise bout induced a rise in muscle *FGF21* mRNA and FGF21 released in the circulation that was further increased 3h in the recovery period in both healthy subjects and patients with T2D[52]. Although the authors did not address it, there seemed to be a trend towards overall decreased expression of muscle FGF21 in the diabetic group. Unfortunately, the study design did not take into account the quantification of the active form of FGF21, but rather its total form. That said, it was demonstrated previously that exercise did not alter the active/total form ratio of FGF21 released into the circulation[138]. The study of FGF21 secretion from isolated hSkMCs in the context of T2D would help validate some of the findings in muscle expression and release into the circulation of this myokine.

**Irisin (FNDC5)**

In 2012, Boström et al. first characterized irisin as an exercise-induced hormone that mediates the browning of WAT[139]. As Perakakis et al. thoroughly reviewed at the beginning of 2017, irisin plays a role in glucose homeostasis, notably by ameliorating IR[80]. The precursor mRNA for irisin, *FNDC5*, is mostly expressed in muscle tissues. In fact, it was shown in humans that *FNDC5* is present up to more than 200-fold in skeletal muscle cells in comparison to adipocytes[140]. This group also found that muscle mRNA expression and circulating irisin levels associated negatively with the status of IR and obesity. Others also found that irisin levels are inversely correlated with the prevalence of T2D and that elevated serum irisin is associated with a reduced risk of developing T2D[141]. In hSkMCs from patients with T2D, study of the suppressive effect of glucose on the expression of *FNDC5* has suggested that hyperglycemia negatively regulates *FNDC5* mRNA expression[93]. Strikingly, secreted levels of irisin from hSkMCs of patients with T2D were found to be higher than in healthy hSkMCs. That said, it was also shown that serum irisin correlates positively with IR, even after adjusting for BMI to exclude obesity related factors[92,94]. Results for either circulating or skeletal muscle irisin levels measured in humans differ greatly between studies and many contradictions can be found in the literature regarding their implication in metabolic disease, as Sanchis-Gomar et al. highlighted in 2014. To this end, Albrecht et al. tested several commercial irisin ELISA kits and found them highly unspecific, which could account for the discrepancies in studies of this myokine[142]. To solve this problem, Jedrychowski et al. used mass spectrometry to measure irisin levels in the plasma of subjects before and after aerobic exercise[143]. They found a significant increase in irisin levels after exercise, thereby suggesting its involvement in the beneficial effects of physical exercise in muscle energy metabolism. Irisin treatment *in vitro* was shown to increase oxidative capacity in muscle while inducing the expression of several metabolic genes involved in mitochondrial biogenesis without activating an inflammatory

response[78,79]. Also, in a HFD-induced rat model of IR, irisin administration improved insulin sensitivity and promoted weight loss, while exercise induced an increase in circulating levels of irisin and similar metabolic improvements[51]. Since irisin is a contraction-induced myokine with overall beneficial effects on energy metabolism, a comparison in the secretion of this protein in response to muscle contraction in patients with T2D and healthy subjects would help better understand how it influences muscle metabolic defects.

**Apelin**

Apelin is a cytokine that has recently been identified as a myokine in a human exercise study showing that muscle from obese individuals submitted to a chronic exercise training regimen had increased apelin mRNA[55]. HSkMCs from these subjects cultured *in vitro* secreted apelin significantly, confirming apelin as a new contraction-induced myokine. Since the increase in muscle *apelin* mRNA correlated with insulin sensitivity improvements from exercise, but that its levels were not increased in plasma, the authors proposed that apelin may act in an autocrine/paracrine fashion on muscle. Recently, apelin expression in muscle was found to be upregulated by chronic exercise in mice, and apelin secretion was increased after *in vitro* EPS of hSkMCs[77]. Vinel et al. also demonstrated that apelin promoted muscle hypertrophy through AMPK activation, as well as mitochondrial biogenesis (increased mitochondrial DNA) and enhanced mitochondrial function (enhanced citrate synthase and aconitase activities and improvements in mitochondrial morphology through increased cristae formation). These findings support the notion that apelin may act in an autocrine/paracrine fashion to induce muscle adaptations to exercise. Apelin treatment in HFD mice improved insulin sensitivity by increasing mitochondrial biogenesis through the AMPK pathway, thereby ameliorating complete fatty acid

oxidation and reducing the production of acylcarnitines in skeletal muscle[76]. Muscle apelin levels in rodent models of T2D were shown to be decreased in comparison to healthy controls[144]. However, results demonstrated that basal plasma apelin levels were higher in patients with obesity and T2D compared to healthy controls. Dray et al. also showed a positive relation between apelin levels in plasma and insulin, glucose and HbA1c levels in humans, supporting the idea that this myokine is increased in the context of T2D[144]. Fasshauer and Bluher proposed that this increase in circulating apelin in the context of obesity and T2D might be a sign of apelin resistance[145]. Apelin is also expressed and secreted by adipocytes, and circulating apelin levels were found to be elevated in the context of obesity[146]. Since apelin secretion is regulated by the presence of insulin, impaired insulin sensitivity leading to hyperinsulinemia could lead to increased plasma apelin in patients with T2D. Further explorations of apelin secretion in skeletal muscle in the context of T2D are needed to better assess the role of this myokine in the development of muscle IR.

**Myonectin – C1q tumor necrosis factor α-related protein isoform 5 (C1QTNF5)**

Serum levels of myonectin were shown to be elevated in rodent models of diabetes[82]. Increased circulating myonectin correlated with lower mitochondrial DNA (mtDNA) content in L6 rat myocytes, drawing a link with mitochondrial dysfunction, while myonectin treatment resulted in improved glucose uptake through activation of the AMPK pathway. Higher myonectin mRNA expression might be a compensatory mechanism for the depleted mitochondrial content in skeletal muscle affected with IR. It was shown that aerobic exercise decreased myonectin levels in plasma, which correlated with the increase in insulin sensitivity and the improvement in mtDNA content in leukocytes from blood samples[57]. Unfortunately, muscle mtDNA and insulin sensitivity were

not measured in these participants. Further research would be necessary to assess the definite role of myonectin in promoting IR and/or mitochondrial dysfunction in muscle in the context of T2D.

**Leukaemia inhibitory factor**

Leukaemia inhibitory factor (LIF) has been shown to promote myoblast proliferation. Broholm et al. demonstrated an increase in LIF secretion by cultured hSkMCs after EPS[147]. It was also shown that LIF is increased in human muscle after exercise, but not in circulation, suggesting this myokine is most likely a regulator of myoblast proliferation by acting in an autocrine fashion. The same group investigated whether muscle LIF and LIF receptor (LIFR) mRNA and protein content would be different in patients with T2D in comparison to healthy subjects. They found that both LIF and LIFR levels were increased in muscle and myoblasts from patients with T2D in comparison to healthy controls[98]. LIF treatment in hSkMCs from patients with T2D did not result in increased proliferation or STAT3 signalling as in cells from healthy subjects, suggesting that the signalling pathways of this myokine are altered in this context. However, another study in mice showed that LIF increased glucose uptake in muscle independently of IR status[83]. In a study focusing on cardio metabolic disease, interval training induced an increase in LIF muscle expression, thereby reversing muscle atrophy linked to myocardial infarction[54]. Since LIF is induced by muscle contraction, it would be interesting to see how this myokine is secreted in patients with T2D during exercise for the improvement of muscle metabolic functions.

**Myostatin**

Myostatin is a myokine known to influence skeletal muscle growth and to be involved in glucose homeostasis, as it stimulates glucose uptake and oxidation through the AMPK pathway[81].

However, in mice fed a HFD, suppression of myostatin by peptibody leads to increased insulin sensitivity through an increase in Akt signalling, as well as glucose transporter GLUT4 expression in muscle[148]. The peptibody treatment also inhibited macrophage infiltration in adipose tissue and expression of pro-inflammatory cytokines in muscle. This study also showed a negative regulation of irisin by myostatin, which could explain the positive metabolic effects of myostatin inhibition in skeletal muscle. Myostatin inactivation increased fatty acid oxidation by improving mitochondrial function in muscle of mice on HFD[149]. However, myostatin deletion was also shown to lead to impaired mitochondrial function and decreased mitochondrial content in a mouse knockout of this myokine[150]. Plasma myostatin levels were found to be significantly lower in patients with T2D in comparison to insulin sensitive subjects[94]. Interestingly, a negative correlation between myostatin and irisin levels was detected in the circulation of patients with T2D, but not in their insulin sensitive counterparts. Contradictorily, in three different groups of obese subjects: patients with T2D, prediabetes and insulin sensitive individuals, an increase in plasma myostatin was shown relative to diabetic status[97]. This last finding suggests that there might be a mechanism of compensation in the skeletal muscle of patients with T2D to improve the impaired glucose metabolism by increasing the production of myostatin. Myostatin also positively correlated with factors predictive of T2D such as fasting plasma glucose and IR, advocating that it might be involved in the pathogenesis of T2D. The authors highlighted that some of the studies presenting a negative relation between T2D and circulating myostatin involved long standing patients with T2D that had been administered antidiabetic drugs for some time, which might influence muscle myostatin secretion. In another study, while only slight differences were found for myostatin levels in the plasma of patients with T2D compared to insulin sensitive subjects, in skeletal muscle, *myostatin* mRNA levels were significantly higher in patients with T2D[96]. In

severely obese patients, myostatin secretion from isolated myoblasts, expression in skeletal muscle and circulating levels were all upregulated in comparison to healthy subjects and correlated positively with IR[95]. What is particularly interesting with myostatin is that its muscle mRNA expression is downregulated by resistance and aerobic exercise[151]. Further investigations are needed to establish more clearly how myostatin function and/or signalling is altered in the context of T2D in skeletal muscle.

### Secreted protein acidic and rich in cysteine

Secreted protein acidic and rich in cysteine (SPARC) is a contraction-induced myokine that has been shown to activate the AMPK pathway in rat muscle cells, influencing glucose metabolism independently of insulin action[85]. In hSkMCs obtained from healthy subjects that followed a strength training intervention, *SPARC* mRNA was found to be increased with regular exercise[152]. However, in db/db mice, plasma and skeletal muscle levels of SPARC were significantly increased, as well as muscle *SPARC* mRNA content[102]. Similarly, in humans, plasma levels of SPARC were found to be positively correlated to BMI, fasting plasma insulin, triglycerides and HOMA-IR, suggesting a role for this myokine in the pathogenesis of T2D[101]. Since SPARC is believed to have a positive effect on muscle energy metabolism, it seems that its signalling in muscle of patients with T2D is impaired.

### β-aminoisobutyric acid.

β-aminoisobutyric acid (BAIBA) is another exercise-induced myokine that acts on skeletal muscle to prevent T2D related metabolic defects. It was demonstrated that BAIBA treatment reduced IR and increased fatty acid oxidation in both C2C12 mouse myocytes and skeletal muscle of mice on

HFD[153]. Also, in a mouse model of T2D, BAIBA treatment improved IR and reduced fasting blood glucose[154]. In wild-type mice, chronic exercise significantly increased BAIBA secretion from skeletal muscle and plasma levels of this myokine[155]. To our knowledge, no study has investigated the secretion and/or expression levels of BAIBA in skeletal muscle of patients with T2D in comparison to healthy subjects.

**Angiopoietin-like 4**

Angiopoietin-like 4 (ANGPLT4) expression is increased after an acute exercise bout in mouse skeletal muscle in relation with the activation of the AMPK pathway[84]. This study also showed that physiological concentrations of ANGPLT4 treatment in C2C12 cells increased mitochondrial function. Nevertheless, it was demonstrated that genetic inactivation of ANGPLT4's ability to inhibit lipoprotein lipase in human improved glucose homeostasis and decreased overall risk of developing T2D[156]. It was also found that plasma ANGPLT4 levels were increased in subjects with impaired glucose tolerance and correlated positively with T2D markers such as HbA1c and HOMA-IR[100]. In addition, muscle *ANGPLT4* mRNA was increased in a mouse model of T2D[99]. In hSkMCs, *ANGPLT4* mRNA was found to be significantly expressed at rest and increased by treatment with long-chain fatty acids in a PPAR-δ-dependant manner[157]. This group found that plasma ANGPLT4 was likely not involved in muscle lipid breakdown although it did correlate with muscle *ANGPLT4* mRNA expression. Nonetheless, ANGPLT4 produced locally in skeletal muscle regulated WAT lipolysis. Recently, it was found that muscle *ANGPLT4* mRNA and plasma levels of this myokine were increased after acute exercise and further increased 3h into recovery in humans, while in the context of T2D, levels of muscle *ANGPLT4* mRNA seemed to be higher in all conditions[52]. This myokine involved in the energy metabolism adaptations of exercise seems

to have altered signalling in patients with T2D, but the implications of this alteration are not known as of yet.

**Exploiting myokines for the treatment of type 2 diabetes**

As discussed in the present review, in the context of T2D, the expression and production of myokines from skeletal muscle is altered (Table 1). Some myokines are upregulated, while others are downregulated. Also, the changes in myokines released in the circulation does not always reflect the expression of these peptides in skeletal muscle and *vice-versa*. A number of myokines improve muscle energy metabolism (i.e. insulin sensitivity, glucose uptake, mitochondrial function, fatty acid oxidation, etc.). Since T2D translates to impaired muscle energy metabolism, this raises the question: is altered myokine secretion involved in the development of metabolic defects during T2D? Why are myokine expression and secretion altered in the context of T2D? Multiple hypotheses could explain this alteration: physical inactivity in patients with T2D could modify their myokine profile, a hyperglycemic environment could regulate their expression and secretion within muscle or systemic inflammation could interfere with myokine signalling due to the secretion of cytokines by adipocytes and macrophages. Another hypothesis that has been brought forward by many research groups is the concept of myokine resistance, or an impaired response to myokine signalling in skeletal muscle that could lead to an alteration of their expression and secretion within muscle tissue. This particular hypothesis could help explain why certain myokines with a positive effect on energy metabolism (i.e. IL-6, IL-13, irisin, FGF21, SPARC, ANGPTL4, etc.) are upregulated in the context of T2D. The signalling pathways of these myokines might be impaired in patients with T2D or their upregulation might be a compensatory mechanism for the metabolic defects occurring in skeletal muscle.

*Table 1. Secretion of myokines is altered by muscle contraction, regular exercise and T2D.*

| Myokine | Influence on muscle energy metabolism | Regulation by muscle contraction | Regulation by chronic exercise | Muscle levels in IR | Plasma levels in IR |
|---|---|---|---|---|---|
| **IL-6** | ↑ glucose metabolism[62]<br>↑ fatty acid oxidation[59,61,62]<br>↓ glucose metabolism chronic exposure[63] | ↑ muscle protein[43]<br>↑ mRNA and plasma protein[52] | | ↑ protein from hSkMCs[86] | ↑[87] |
| **IL-8** | | ↑ mRNA[118] and protein from EPS[118,120]<br>↑ mRNA[119] | | ↑ protein from hSkMCs and muscle[86,122,123] | ↑[121]<br>=[124] |
| **IL-10** | ↑ insulin sensitivity[64]<br>↑ glucose metabolism[65] | = plasma protein[53] | ↑ plasma protein[53] | | ↓ secretory response[125] |
| **IL-13** | ↑ insulin sensitivity[67]<br>↑ glucose uptake[66] | ↑ protein[120] | | ↓ protein from hSkMCs<br>= mRNA[66] | ↓[66] |
| **IL-15** | ↑ oxidative capacities[68] | ↑ mRNA[127,128] | ↓ plasma protein[88] | ↑ protein from hSkMCs[86] | ↑ [88] |
| **IL-18** | ↑ lipid oxidation[70] | | | ↓ muscle mRNA in IR[70] | ↑[89] |
| **Musclin** | ↓ glucose uptake and insulin sensitivity[133] | | | ↑ protein and mRNA[134] | ↑[132] |
| **FGF21** | ↑ insulin sensitivity[72,136]<br>↑ fatty acid oxidation[136] | ↑ plasma protein, muscle mRNA and protein[52,137] | | ↑ protein[91]<br>↑ mRNA[90] | =[90] |
| **Irisin** | ↑ oxidative capacities and mitochondrial biogenesis[78,79]<br>↑ insulin sensitivity[51,80] | ↑ plasma protein[51,143] | | ↓ mRNA[140]<br>↑ protein from hSkMCs[93] | ↓[140,141]<br>↑[92,94] |
| **Apelin** | ↑ insulin sensitivity[76], fatty acid oxidation and mitochondrial biogenesis[76,77] | ↑ protein from hSkMCs[77] | ↑ muscle mRNA[55,77] | ↓ protein[144] | ↑[144] |
| **Myonectin** | ↑ glucose uptake[82] | ↓ plasma protein[57] | | | ↑[82] |

| | | | | | |
|---|---|---|---|---|---|
| **LIF** | ↑ glucose uptake[83] | ↑ protein from hSkMCs and muscle, = plasma[147] | ↑ muscle mRNA[54] | ↑ protein from hSkMCs and muscle[98] | |
| **Myostatin** | ↑ glucose uptake and oxidation[81] | ↓ muscle mRNA[151] | | ↑ muscle mRNA[95,96] and protein from hSkMCs[95] | ↑[95-97] ↓[94] |
| **SPARC** | ↑ glucose uptake[85] | | ↑ hSkMCs mRNA[152] | ↑ muscle mRNA and protein[102] | ↑[101,102] |
| **BAIBA** | ↑ insulin sensitivity[153,154] and fatty acid oxidation[153] | | ↑ muscle and plasma protein[155] | | |
| **ANGPTL4** | ↑ mitochondrial function[84] | ↑ muscle mRNA[52,84], plasma protein[52] | | ↑ muscle mRNA[99] | ↑[100] |

*Legend: ↑ upregulated, ↓ downregulated, = unchanged, numbers in (…) refer to references in the text.*

Ever since the discovery of myokines and the regulation of their secretion by muscle contraction, numerous research groups have proposed the possibility of using these factors for the treatment of metabolic diseases. In a review published at the beginning of 2018, J.Y. Huh proposes that myokines released by muscle could be a mechanism of adaptation of this organ to the increase in glucose demand during contraction. This theory could explain in part why myokine secretion is altered in patients with T2D since fasting blood glucose and insulin levels are elevated in these conditions. Another interesting idea brought forward by this author is the synergy in regulation of myokines secreted by muscle cells. Many myokines have been proven to be regulated in part by other myokines, for example myostatin and irisin whose levels are usually inversely correlated, especially in the context of T2D. This cooperative action of myokines during muscle contraction and exercise could explain the complexity of the effect it has on energy metabolism, specifically in the context of T2D. The altered secretion of one myokine could create a butterfly effect on other myokines resulting in the alteration of skeletal muscle glucose and lipid metabolisms.

As mentioned before, the relationship between sedentary behavior and the prevalence of T2D has been explored by many groups and a clear correlation has been drawn between lack of physical exercise and the development of IR, especially in the context of obesity[32]. Also, the levels of many myokines have been studied in individuals before and after following a training program to observe variations in their secretion in response to regular exercise. Some myokines are found to be upregulated in trained individuals in comparison to sedentary subjects, while other myokines are downregulated in the context of regular physical activity. For example, chronic exercise upregulates IL-6, IL-10, FGF21, irisin, LIF, BAIBA and apelin, while it downregulates myostatin, myonectin and musclin[51-57,155] (Fig. 1). This suggests that the alteration in myokine secretion in patients with T2D could be explained by the lack of physical exercise in their lifestyle. To verify

this hypothesis, the skeletal muscle myokinome, as proposed by Nikolić et al. in 2017, of patients with T2D should be investigated to find a potential correlation between the T2D profile and their level of physical activity.

Another factor that could explain the differences in myokine secretion between healthy subjects and patients with T2D is the state of chronic low-grade inflammation. Since myokines are cytokines, some of them are also secreted by other tissues and act as inflammatory factors when released in the circulation by immune cells. Therefore, it is difficult to determine if the alteration in myokine secretion in plasma/serum is due to systemic inflammation or altered skeletal muscle secretion. As reviewed in 2017 by Kalinkovich and Livshits regarding obesity and sarcopenia, some myokines are exacerbated by adipose tissue inflammation, which results in an even more important inflammatory response through the secretion of these factors by skeletal muscle (MCP-1, myostatin. TNF-α, IL-6, IL-10 and IL-1β)[158,159]. It has been suggested that certain myokines induced by inflammation, including irisin, IL-8, IL-15 and FGF21, might play a role in limiting the negative effects of this state on skeletal muscle energy metabolism by enhancing oxidative capacity[86,158]. This mechanism could serve as a balance for the reestablishment of a functional energy metabolism in muscle previously altered by adipose tissue signalling in the context of systemic inflammation. Some myokines are also decreased in chronic low-grade inflammation, such as IL-13[66], but the mechanisms resulting in the reduction of expression and/or secretion of these myokines remains to be fully understood. Another important source of pro-inflammatory factors in the context of obesity and T2D is intermyocellular adipose tissue (IMAT) and perimuscular adipose tissue (PMAT)[160]. These skeletal muscle fat deposits expand in the context of obesity and it is suspected that they are the source of pro-inflammatory factors involved in chronic low-grade inflammation such as MCP-1[86,160]. Since this form of adipose tissue is

embedded in the muscle tissues, it is difficult to differentiate the effect of the factors secreted by the adipocytes from the metabolic changes induced by factors secreted by the myocytes. IMAT and PMAT could be involved in the development of skeletal muscle IR and/or mitochondrial dysfunction in the context of T2D through altered adipose tissue signalling[161]. Conditioned media from adipocytes isolated from the IMAT of patients with obesity was shown to reduce insulin signalling and glycogen synthesis in hSkMCs, supporting a role in the development of muscle IR[162]. To our knowledge, this study is the only one to have found a direct causal link between IMAT and the incidence of developing of muscle IR. Further observation of the crosstalk between these fat deposits and hSkMCs would be required to further establish the contribution of IMAT and PMAT in muscle metabolic defects found in patients with T2D.

To avoid speculation about the factors inducing changes in myokine secretion in patients with T2D, new studies should be conducted using alternative approaches to the identification of these factors and how they act on skeletal muscle energy metabolism. By measuring the output of molecules from skeletal muscle through the collection of conditioned media from cultured hSkMCs, through microdialysis or muscle targeted blood samples, peptides, metabolites and RNAs collected can be identified as originating directly from muscle and not from other tissues. Then, considering the availability of many new high-throughput methods to collect and analyse data nowadays, conducting a proteomics study on the secretome of skeletal muscle from patients with T2D in comparison to healthy subjects seems essential. This analysis has been achieved by other groups before, but not with a focus on all signalling molecules[163-167]. This approach could allow the visualisation of protein expression networks in clusters, facilitating the formation of new links in the expression or secretion of certain myokines with other myokines. After identifying synergistic effects between two or more myokines, further studies could be conducted with a

targeted approach to characterize that relationship and better understand how it influences the development of IR in muscle. Eventually, these steps in validating the pertinence of myokines as a target for the treatment of T2D could lead to the development of new therapies based on either physical exercise targeting the secretion of one or multiple myokines known to be altered in the context of T2D or drugs simulating this effect (i.e. drugs that improve the secretion of one or multiple myokines linked to the development of IR). In fact, a study comparing moderate continuous intensity training (MICT) with high intensity interval training (HIIT) in patients with T2D showed a greater improvement in glucose homeostasis and $VO_2$max in the HIIT group following the training protocol, even with a reduced training volume and energy expenditure compared to the MICT group[168]. The molecular mechanisms underlying this increased effectiveness of HIIT for the management of T2D in comparison to MICT are not understood. Therefore, it is a possibility that HIIT induces the secretion of certain factors, from skeletal muscle that mediate these beneficial effects on muscle metabolism and whole-body insulin sensitivity.

**What about the other factors in the skeletal muscle secretome?**

At the beginning of 2018, Whitham et al. demonstrated the existence of a skeletal muscle to liver crosstalk during physical exercise mediated through the secretion of extracellular vesicles (EVs)[169]. The EVs were observed to be induced by muscle contraction and transported to the liver, where their content was delivered to hepatocytes. This research group also analyzed the contents of these vesicles to identify the myokines they contained and found many proteins already known to be secreted by hSkMCs. This suggests that skeletal muscle can act as an endocrine organ by secreting factors in a non-traditional manner, meaning not through soluble proteins in circulation, during contraction to influence whole-body energy metabolism. When cultured *in vitro*, biopsy-

derived hSkMCs maintain the metabolic characteristics of their donors (i.e. metabolic flexibility, IR and lipid content)[170,171]. Consequently, muscle cells from patients with T2D maintain their diabetic phenotype *in vitro* (i.e. IR, impaired mitochondrial functions, incomplete fatty acid oxidation, etc.)[172-178]. To our knowledge, the proteome contained in skeletal muscle EVs of patients with T2D has not been compared to the one from subjects who are insulin sensitive. Since the secretome of hSkMCs from patients with T2D show altered myokine secretion[86], we hypothesize that the proteins contained in EVs secreted by IR muscle will also be different from insulin sensitive muscle. A study of this particular proteome could allow a better understanding of the molecular mechanisms leading to IR and mitochondrial dysfunction during T2D. It is possible that hSkMCs that display IR and impaired fatty acid oxidation release different factors in their EVs during contraction which mediate an impaired energy metabolism response in the surrounding cells/tissues. In the context of rheumatoid arthritis, another chronic non-communicable disease related to immunometabolism, it has been shown that EVs can be released locally in the musculoskeletal joints to induce acute inflammation[179]. These EVs contain miRNAs that can either induce or inhibit the release of pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β[180]. It is thus possible to hypothesize that similar miRNAs could be released in EVs from skeletal muscle in the context of obesity and/or T2D and could specifically regulate the secretion of certain myokines in surrounding muscle cells, leading to the development of muscle IR and/or mitochondrial dysfunction. It might be important to include in future proteomics studies of skeletal muscle from patients with T2D a component taking into account EVs. This would allow a better understanding of the potential role of these different skeletal muscle signalling factors in the development of T2D associated muscle metabolic defects.

In addition, it has been shown by many groups that certain metabolites, namely intermediates of fatty acid metabolism, are involved in the development of metabolic defects in skeletal muscle associated with T2D. Certain miRNAs specific to skeletal muscle have also been suggested to be involved in the diabetic phenotype, especially in relation to IR. Many myo-miRNAs (myomirs) have been shown to be either induced or decreased in the context of T2D and these myomirs were identified to be targets of numerous different cell signalling cascades[181]. This points to a role of miRNAs in skeletal muscle signalling leading to the development of T2D. Therefore, these molecules should not be ignored during the analysis of the muscle secretome to better understand how IR and mitochondrial dysfunction occur in patients with T2D. In fact, either myomirs and/or metabolites could be involved in the altered secretion of myokines found in patients with T2D. A study showed that muscle cells could secrete exosomes containing miRNAs that regulated the expression of differentiation factors in myocytes[182]. These miRNAs could then also potentially regulate the expression of certain myokines, as discussed earlier regarding this method of regulation for cytokine production in the context of arthritis. Alterations in the production of these factors could lead to the altered secretion of myokines in skeletal muscle in the context of T2D. Considering this hypothesis, it would be relevant to observe how these myomirs can be induced by muscle contraction to eventually exploit their role on skeletal muscle energy metabolism for the treatment of T2D.

**Conclusion**

A lot remains to be determined concerning myokine secretion and expression in the context of T2D. Many of the myokines found to be dysregulated in the circulation of muscle of patients with T2D have yet to be identified as secreted by this tissue. Also, some myokines known to

influence muscle energy metabolism still have not been characterized in skeletal muscle affected with IR and/or mitochondrial dysfunction. By establishing the potential candidate myokines induced by exercise that could mediate a maximal attenuation of the muscle metabolic defects found in patients with T2D, exercise programs and/or pharmaceutical treatments could be developed to target the secretion of these molecules. This could serve as a new method of management of T2D focused on skeletal muscle metabolism."

This marks the end of the previously published literature review focused on myokines and their implications in T2D and muscle metabolic characteristics, while the next sections of the introduction follow.

**An In Vitro Model of the Muscle *Interstitium* During Contraction**

Since myokines can be secreted by other tissues, measurement of their secretion in plasma or serum does not adequately represent skeletal muscle signaling. That said, when isolated from muscle biopsies of patients with T2D and cultured *in vitro*, hSkMCs maintain their IR and mitochondrial dysfunction[172,173,175,177]. Although these isolated muscle cells can be differentiated into myotubes that resemble mature muscle cells, myotubes do not possess the maturity of muscle fibers and they lack the crosstalk from surrounding tissues. Nevertheless, this model using samples obtained directly from clinical participants is the best known model to date for the study of myokines secreted specifically by skeletal muscle during T2D[183].

In addition, myotubes can be submitted to electrical pulse stimulations (EPS), which mimic muscle contraction *in vitro*[184]. EPS has been shown to induce metabolic adaptations similar to those observed in muscle after exercise, while also increasing myokine secretion[120,185]. Most studies using EPS with hSkMCs employed long durations of stimulations (4-48h) at low frequencies (≤2Hz) resulting in improved oxidative functions, mitochondrial biogenesis, glucose uptake, insulin sensitivity and increased release of myokines in the cell culture supernatant[120,185-189]. That being said, these stimulation conditions do not translate to muscle contraction *in vivo*, as the stimulus is considerably longer than an average bout of exercise would last. Nikolić et al. observed that treatment of hSkMCs with acute EPS (30 V, 200 ms impulse trains, 100 Hz, pulse every fifth second) resulted in increased glucose uptake, lactate production and decreased ATP and phosphocreatine contents proportional to stimulation duration (5-60 minutes), which corresponds to muscle metabolic adaptations to acute exercise[184]. On the contrary, chronic EPS treatment (24–48h duration, 30 V, 2 ms impulse trains, 1 Hz) improved oxidative capacities, resulting in increased

glucose uptake and oxidation, as well as complete fatty acid oxidation, which represent muscle adaptations to chronic exercise[184].

Early on during my PhD research, we attempted to replicate the acute EPS conditions of Nikolić and colleagues and found that they caused cell death with the stimulation apparatus we were using. Since the first EPS conditions[184] we tested resulted in cell death in both hSkMCs and L6 rat muscle cells, we decided to reproduce another protocol with milder voltage and frequency that was used with the exact same apparatus as ours. Indeed, Li et al. treated C2C12 mouse muscle cells with 1h of EPS at 20V, 1Hz frequency with 24ms impulses every 976ms (24ms on the second), resulting in increased cell surface GLUT4 localization through AMPK signal transduction, and downstream TBC1D1 and AS160 (TBC1D4) activation by phosphorylation[190]. C2C12 myotubes have a greater EPS contractile activity than hSkMCs because of their more rounded morphology[191], therefore it was crucial to validate these conditions in human myotubes. To our knowledge, no other research team had attempted to replicate these acute muscle cell contraction conditions in hSkMCs to study molecular signaling events *in vitro*.

**Markers of Muscle Metabolic Adaptations to Exercise**

The AMP activated protein kinase (AMPK) pathway is central to muscle signaling during exercise, since it serves as an energy sensor responding to the rise in AMP/ATP ratio that occurs during muscle contraction[192]. When phosphorylated in muscle in response to contraction, AMPK induces downstream signaling to inhibit anabolic processes (e.g. phosphorylation of acetyl-CoA carboxylase (ACC) to block lipid synthesis) and activate catabolic pathways (e.g. induction of GLUT4 translocation to the plasma membrane). Therefore, muscle AMPK and ACC phosphorylation, as well as GLUT4 translocation serve as adequate markers of acute exercise simulation in EPS experiments. Another pertinent marker of exercise adaptations downstream of

AMPK is peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1$\alpha$). This co-activator is central to fiber-type switching in skeletal muscle to enhance oxidative functions by favoring slow-twitch fibers via remodeling of mitochondrial dynamics[193]. Consequently, PGC1$\alpha$ expression in response to EPS relates to adaptations to chronic exercise. Another group of proteins that are central to the cellular adaptions following muscle contraction are the sirtuins. SIRT1 is a nuclear and cytosolic protein that is essential to survival and has many targets related to exercise adaptations such as PGC1$\alpha$[194]. On the whole, an increase in SIRT1 expression leads to a shift from glycolytic to a more oxidative muscle metabolism, representing adaptations to exercise training. That being said, an increase in SIRT1 expression can be observed in muscle as soon as 2h in the recovery period after sprint exercise[195]. SIRT3, on the other hand, is a mitochondrial inner membrane protein known to be activated by oxidative stress, as it enhances the mitochondrial respiratory chain efficiency by acting directly on different components of the electron transport chain complexes, thereby minimizing ROS production[196]. Increased SIRT3 protein in muscle after exercise training indicates this protein is a marker of long-term muscle metabolic adaptations to regular exercise and interestingly, this regulation of SIRT3 was found to be AMPK-dependent[197]. Finally, calcium/calmodulin-dependent kinase II (CaMKII) signal transduction is an important modulator of glucose uptake into muscle tissue and cells during contraction both *in vivo*, and in an *in vitro* model of acute exercise[198].

**Other Factors of the Muscle Secretome**

As mentioned previously, skeletal muscle can secrete other factors than myokines during contraction. EVs comprise exosomes (20-100nm in diameter), microvesicles (100nm-1μm in diameter) and apoptotic bodies (1-5μm in diameter)[199], but for our purposes, these last vesicles are omitted when using the term “EV”. EVs are membrane bound vesicles containing a bioactive

cargo. Exosomes originate from the inward budding of the endosomal membrane, while microvesicles can be formed from the direct outward budding of the plasma membrane[199]. EV secretion in plasma was found to be increased in response to exercise, suggesting these molecules could play a role in mediating the beneficial effects of exercise[200]. Since these EVs were measured in circulation, it is also possible that they originated from other tissues than skeletal muscle. However, acute exercise was shown to induce the release of EVs directly from skeletal muscle (arteriovenous balance from a contracting limb) in circulation addressed to and absorbed by the liver[169]. This finding suggests the existence of a crosstalk mechanism between muscle and other tissue(s) using non-traditional protein secretion (other than soluble proteins targeted via their N-terminal sequence) mediated by EVs. Also, others demonstrated the release of EVs containing proteins, miRNAs and metabolites from hSkMCs cultivated *in vitro*, further supporting the potential role of EVs in muscle signaling[182,201,202]. Importantly, some of these miRNAs contained in the EVs were shown to regulate the expression of proteins involved in myocyte differentiation, outlining the role of these vesicles in autocrine/paracrine signaling in muscle[182]. It is possible that exosomes and/or microvesicles could play a role in the development of muscle metabolic defects during T2D. Supporting this hypothesis, exosomes isolated from myotubes treated with palmitate to induce IR were shown to induce alterations in genes linked to muscle differentiation in untreated myotubes[203]. Moreover, some muscle miRNAs (myomirs) were found to be altered in patients with T2D, promoting the idea of their role in the pathogenesis of this disease[181].

**Different Types of Exercise for the Management of T2D**

Recommendations in terms of physical activity from Diabetes Canada are broad: minimum 150 minutes of aerobic exercise per week, along with two resistance training sessions. Traditionally, aerobic exercise modalities used in interventions with patients with T2D involved efforts of

moderate intensity (reaching 55-75% of maximal effort capacity as measured by different markers of cardiorespiratory fitness). On the whole, these types of intervention have proven efficient in improving whole-body glucose homeostasis and a combinatory effect can be observed when adding resistance exercise to the intervention[204]. Recently, the use of alternative training regimens to classic moderate intensity continuous training (MICT) has been growing in popularity for the management of chronic metabolic diseases such as obesity and T2D. In fact, it was shown that high intensity interval training (HIIT) was more efficient in improving glucose homeostasis and respiratory capacity ($VO_2$max) in patients with T2D than MICT, even with a reduced training volume[168]. Similarly, functional HIIT in the form of Crossfit™ was shown to be efficient in improving whole-body insulin sensitivity and fatty acid metabolism in patients with T2D while maintaining a very high compliance with the training program[205]. Since lack of motivation and/or time are the most cited barriers to achieving recommended levels of exercise for patients with T2D, these alternative training methods seem like relevant alternatives to MICT[206]. Nonetheless, the molecular mechanisms explaining these differences in metabolic adaptations between different types of training have not been elucidated. It is then possible that myokine secretion could be regulated differently by HIIT than MICT, which could explain in part why HIIT was shown to be more efficient in managing metabolic defects found in T2D.

**The Concept of Exercise Resistance**

On the other hand, some subjects experience no improvements in glucose homeostasis in response to exercise interventions. After a training protocol of 12-16 weeks of aerobic MICT in a cohort of around one hundred subjects with pre-diabetes or T2D, roughly one third of participants showed no improvements in glucose control[207]. Similar results were found in women with impaired glucose tolerance after a 12-week HIIT or resistance training intervention[208]. This supports the

notion of non-responders to glucose control improvements with different types of training. Our collaborators (Dr. Sparks' group) uncovered part of the molecular mechanisms explaining these discrepancies in exercise response during T2D in the differential expression of genes linked to oxidative capacity at baseline in non-responders to a nine-month training intervention[209]. Many other factors such as duration of T2D, age, genetics and epigenetics contribute to the exercise response in patients with T2D, regardless of exogenous factors such as the type and duration of the exercise intervention[210]. Nevertheless, the molecular mechanisms explaining the occurrence of non-responders to improved glucose homeostasis after exercise interventions are not completely understood and to our knowledge, the myokine profiles of responders and non-responders has not yet been compared after training. Altered myokine secretion in response to acute and chronic exercise could therefore be a factor contributing to the impaired improvements of glucose control in patients with T2D non-responsive to exercise training.

## Statement of Objectives

The link between physical activity levels, myokine secretion and glucose homeostasis in patients with T2D remains to be completely understood. Since myokine secretion is altered in T2D and this chronic disease is strongly associated with sedentary behavior, we hypothesized that changes in physical activity levels could alter muscle signaling events, namely myokine secretion, in patients with T2D, and these alterations would follow improvements in glucose homeostasis in this patient population. As myokines play a role in muscle metabolic adaptations to exercise, regular physical activity should positively modulate myokine secretion in T2D patients. In the case of certain myokines, a positive regulation would be synonymous of increased levels in circulation or muscle in patients with T2D, whereas other myokines' secretion would be decreased in these patients. This hypothetical mechanism resulting from muscle contraction could explain in part the beneficial effects of a more active lifestyle and reduced sedentary behavior in patients with T2D for the management of its symptoms.

**Specific objectives:**

1) Characterize the regulation of peripheral myokine secretion in subjects with or without obesity in response to an acute bout of aerobic exercise.
2) Compare peripheral cytokine (candidate myokines) secretion in subjects with CAD with or without T2D in response to two different training modalities.
3) Study the effect of chronic exercise on myokine secretion in subjects with obesity and individuals with both obesity and T2D with or without resistance to exercise training.

# Chapter 2

# Plasma Myokine Concentrations After Acute Exercise in Non-obese and Obese Sedentary Women

Our aim was to study peripheral myokine secretion in the context of individuals with or without obesity performing an acute bout of aerobic exercise to determine whether the occurrence of obesity could impact acute myokine release in circulation. This study was completed in collaboration with Dr. Lauren Sparks' team at Advent Health's Translational Research Institute for Metabolism and Diabetes in Orlando, Florida. Dr. Sparks and her team (led by Dr. Steven R. Smith) graciously accepted to share blood samples from a clinical trial named Columbus completed in 2014 (NCT01911091). We used plasma samples collected at various time-points following an acute bout of moderate intensity aerobic exercise, and we were able to look at peripheral myokine levels in these participants over the 24h period of sampling pre- and post-exercise. We assessed the regulation of FGF21, SPARC, IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18 in circulation in female subjects with or without obesity in response to an acute bout of cycling exercise.

This study was published in the journal Frontiers in Physiology as a part of a special research topic: The Role of the Muscle Secretome in Health and Disease.

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**Plasma Myokine Concentrations After Acute Exercise in Non-obese and Obese Sedentary Women**

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## Abstract

Exercise and physical activity levels influence myokine release from skeletal muscle and contribute to circulating concentrations. Indeed, many myokines, including interleukin (IL-) 6, IL-15, secreted protein acidic rich in cysteine (SPARC) and fibroblast growth factor (FGF) 21 are higher in the circulation after an exercise bout. Since these peptides modulate muscle metabolism

and can also be targeted towards other tissues to induce adaptations to energy demand, they are of great interest regarding metabolic diseases. Therefore, we set out to compare, in 6 women with obesity (BMI ≥ 30kg/m$^2$) and 5 healthy women (BMI 22-29.9 kg/m$^2$), the effect of an acute bout of moderate-intensity, continuous cycling exercise (60 minutes 60% $VO_2$peak) on the release of myokines (IL-6, IL-8, IL-10, IL-13, IL-15, SPARC and FGF21) in plasma for a 24h time-course. We found that plasma IL-8 and SPARC levels were reduced in the group of women with obesity, whereas plasma IL-13 concentrations were elevated in comparison to non-obese women both before and after the exercise bout. We also found that plasma FGF21 concentration during the 24h following the bout of exercise was regulated differently in the non-obese in comparison to obese women. Plasma concentrations of FGF21, IL-6, IL-8, IL-15 and IL-18 were regulated by acute exercise. Our results confirm the results of others concerning exercise regulation of circulating myokines, while providing insight into the time-course of myokine release in circulation after an acute exercise bout and the differences in circulating myokines after exercise in women with or without obesity.

## Introduction

Since the beginning of the 2000s, skeletal muscle has gained notoriety as an important source of secreted factors such as peptides, RNAs, metabolites and extracellular vesicles that can contain any of these molecules. These factors are significant regulators of whole-body energy metabolism. The peptides secreted by the skeletal muscle are called myokines as many of these peptides are also cytokines (e.g. the interleukins (IL)) (Pedersen et al. 2003). Myokines have been studied for their effect on skeletal muscle metabolism, but also as endocrine effectors of energy metabolism adaptions (Garneau and Aguer 2019, Nielsen and Pedersen 2007).

During exercise and/or muscle contraction, the levels of some myokines in the muscle are greatly increased, and circulating levels of these peptides are also modulated by exercise. For example, after an acute bout of moderate-intensity cycling on a stationary bicycle (70% $VO_2$max), IL-6 and fibroblast growth factor (FGF) 21 expression was increased in skeletal muscle, while serum levels of IL-6, IL-15 and FGF21 were significantly increased (Sabaratnam et al. 2018). The effect of exercise on the secretion of myokines from skeletal muscle locally cannot necessarily be translated to the release of these peptides in the circulation, as reviewed in (Garneau and Aguer 2019). Indeed, some myokines are believed to act directly on skeletal muscle to improve its energy metabolism during contraction. For example, IL-6 increases muscle fatty acid oxidation and glucose uptake in L6 myotubes and human primary muscle cells from *rectus abdominus* muscle (Al-Khalili et al. 2006, Carey et al. 2006), while IL-13 treatment directly stimulates glucose uptake and oxidation in human primary myotubes (Jiang et al. 2013). On the contrary, the mechanism of action of myokines can also require their release in the circulation to be delivered to their target tissue(s). Examples of such mechanisms include the stimulation of fatty acid oxidation and glucose uptake into white adipose tissue by IL-6 during exercise recovery (Knudsen et al. 2017) and the positive effect of IL-13 on both rat and human pancreatic β-cell survival (Rutti et al. 2016). This underscores the different mechanisms of action of myokines depending on their release locally in skeletal muscle (muscle protein and mRNA) and in the circulation (plasma or serum protein). The metabolic functions of myokines draw a link between exercise adaptations and amelioration of muscle and whole-body metabolisms, which is of interest in the context of obesity as it is an important risk factor for the development of type 2 diabetes (T2D) (Muoio and Newgard 2008).

In patients affected with certain chronic non-communicable diseases (e.g. sarcopenia, arthritis, obesity and T2D), circulating myokine levels are altered. These diseases are generally associated

with systemic, low-grade inflammation (Gregor and Hotamisligil 2011). For example, IL-6 and FGF21 were found to be elevated in patients with obesity and correlated with whole-body adiposity (Vozarova et al. 2001, Zhang et al. 2008), circulating IL-8 and SPARC correlated positively with BMI (Kim et al. 2006, Wu et al. 2011), while plasma IL-15 correlated negatively with trunk fat mass and body-fat percentage (Nielsen et al. 2008) but also have been shown to be elevated in the setting of obesity (Perez-Lopez et al. 2018). It is well-established that acute and chronic exercise can modulate circulating myokine levels. However, it is currently unclear if the presence of obesity (BMI >30) can impact the myokine response in sedentary subjects. In this regard, our study design allows us to observe the potential effects of obesity on the variations in circulating myokines following an acute bout of exercise.

Here we evaluate the dynamic and time-dependent changes in plasma myokine concentrations (pre-, immediately after an acute bout of moderate-intensity cycling exercise (0h), 1h, 2h, 3h, 4h, 12h and 24h post-exercise) in sedentary women with and without obesity.

## Materials and Methods

**Participants and Study Design.** A total of 11 women, 18-40 years of age, were included in this study; 6 women were classified as obese (BMI of > 30 kg/m$^2$) and 5 were classified as non-obese (BMI of 22-29.9 kg/m$^2$). Other inclusion and exclusion criteria were as follow: weight stable (<3 kg variations in the last 8 weeks), not involved in any exercise program, willing to stop caffeine and alcohol consumption 48h prior to blood draw, no history of T2D, HbA1c ≤ 6.5% (measured in the obese group only), favorable anatomy for continuous venous blood sampling, no presence of clinically significant abnormalities on ECG, no significant renal, cardiac, liver, lung or neurological disease (controlled hypertension acceptable), no use of drugs known to affect metabolism or body-weight (e.g. orlistat, sibutramine, ephedrine, phenylpropanolamine,

corticosterone), current treatment with blood thinners or anti-platelet medications that cannot be stopped safely, no new onset (<3 months) of oral contraceptives or hormone replacement therapy, no current smokers (>3 months), not currently pregnant or having nursed a child within the last 12 months, no gait problems, no increased liver function tests (AST/ALT/GGT or alkaline phosphatase greater than 2.5 times the upper limit of normal), no in-body metal objects that would interfere with body composition measurements, no New York Heart Association any class heart failure, no history of deep vein thrombosis or pulmonary embolism, no significant varicose veins, no abnormal blood count or blood donations in the last two months, no major surgery of the abdomen, pelvis or lower extremities in the last 3 months, no bariatric surgery or liposuction within the previous 3 years, no cancer, no rheumatoid disease, no bypass graft in limb, no known genetic factor (e.g. Factor V Leiden) or hypercoagulable state, no diagnosed peripheral arterial or vascular disease or intermittent claudication, no peripheral neuropathy, no claustrophobia, no major depression, no presence of an eating disorder or eating attitudes/behaviours that could interfere with the study and no nocturnal urination and/or sleep apnea.

Two days prior to performing the exercise bout, participants underwent a diet stabilization period and were given standard diet composition meals (35% fat, 15% protein and 50% carbohydrates; prepared and pre-packed at the exercise facility). The day before the exercise test, participants came fasted at the facility, underwent all baseline measures, then ate the standardized diet for breakfast and lunch. At night, they consumed a high carbohydrate dinner and snack (70-75% carbohydrate, 12-15% fat, 10% protein), then performed the cycling exercise bout at around 7 am the following morning. Plasma samples from these participants were obtained immediately before (pre-), immediately after (0h) and 1h, 2h, 3h, 4h, 12h and 24h into the recovery period after a fasted acute bout of moderate-intensity continuous exercise. The exercise bout consisted of 60 minutes

of cycling on an upright stationary bicycle (Vision Fitness U40, Wisconsin, USA) at 60% $VO_2$peak. Participants remained fasted for the first 4h of recovery with limited liquid for consumption (600mL) and were fed meals of standard diet composition (35% fat, 15% protein and 50% carbohydrates) after the '4h' blood draw, with limited total liquid consumption of 1000ml. Blood samples were collected with a venous catheter in standard anti-coagulant (EDTA) tubes, centrifuged at 2000 *g* for 10 minutes, aliquoted and stored at -80°C until analyses.

**Body composition, blood analyses and $VO_2$max determination.** Whole-body fat and lean mass were assessed through Dual Energy X-Ray Absorptiometry (DEXA) scans (GE Lunar iDXA whole-body scanner, GE Healthcare, USA). Glucose, insulin and glycated hemoglobin (HbA1c) were measured in fasted blood samples in the clinical chemistry laboratory at either Florida Hospital or onsite at the Translational Research Institute for Metabolism and Diabetes, as previously described (Stephens et al. 2018) to assess the eligibility of the participants. An HbA1c ≤6.5% would have resulted in the exclusion of the participant from the study. Glucose and insulin levels were used to determine HOMA-IR values (HOMA = [(fasting insulin (IU/mL)) × (fasting glucose (mmol/L))]/22.5), which attest of the sensitivity to insulin, therefore of the metabolic health of the participants (Antuna-Puente et al. 2011). All tests were performed during the follicular phase of the participants' menstrual cycle to avoid the confounding effects of the hormonal surges during the other phases on circulating cytokine levels. Aerobic fitness was assessed through maximal oxygen consumption ($VO_2$max) incremental test on a cycle ergometer as in (Costford et al. 2010), and $VO_2$max was reached if the respiratory exchange ratio increased to 1.10 or higher and the participant's heart rate increased to within 10 beats of the age-predicted maximum (208 – (0.7 x age)) or the rate of perceived exertion (RPE) was 17 or over on a scale of 6-20.

**Myokine quantification.** SPARC (also known as osteonectin) and FGF21 were quantified by single plex assays from MesoScale Discovery (R-plex; MSD – Maryland, USA), whereas IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18 were quantified by multiplex assay (U-plex; MSD) in plasma obtained from venous blood samples taken from the arm of the participants. Intra-assay coefficients of variations (CV) for the standards for SPARC, FGF21 and the multiplex were 10.00%, 8.02% and 8.28%, respectively. Samples were measured only once, therefore their intra- and inter-assay CV cannot be calculated. All antibodies, with the exception of SPARC, were validated for target specificity. Information can be found on the datasheet of the different U-plex antibody sets (https://www.mesoscale.com/) in the supplementary materials.

**Statistical analyses.** Data are presented as means ± standard deviation (SD). Anthropometric measures were analyzed by unpaired t-test or Welch's t-test (depending on the standard error values), while all myokine quantifications were analyzed by two-way ANOVA with repeated measures or mixed model analysis (when samples from certain time-points were missing) along with Dunnett's multiple comparison as post hoc tests and area under the curve (AUC) of all time-points were assessed and were analyzed by unpaired t-test or Welch's t-test. All statistical analyses were performed with Prism 8 software (San Diego, California, USA). A p-value $\leq 0.05$ was considered significant.

## Results

### Characteristics of the participants

The characteristics of the participants for the two parts of the study are presented in Table 1. No statistical differences were found between the 2 groups that performed the acute bout regarding age, blood glucose, insulin, insulin sensitivity (HOMA-IR), and $VO_2max$, but the BMI ($p<0.05$), the fat mass ($p<0.05$) and the total lean mass ($p<0.01$) of the women from the obese group were

significantly higher than the non-obese group. The HOMA-IR values in the two groups of non-obese or obese sedentary individuals suggest these participants were insulin resistant.

**Time course of plasma myokine concentrations in response to acute exercise.** In response to an acute cycling bout, FGF21, IL-6, IL-8, IL-15 and IL-18 concentrations were significantly altered in plasma of both obese and non-obese women subjects over 24hrs after the exercise (Fig.1). For IL-6 and IL-18, these myokines increased transiently after the exercise bout and returned to pre-exercise values in the circulation within 24h into the recovery period in both subject groups. On the contrary, IL-8 levels in plasma decreased gradually over the 24h following the exercise bout, reaching significance at the 4h and 24h time-point, while IL-15 levels increased continuously to reach levels higher than basal after 12h into recovery. No significant effect of the exercise bout was detected on plasma levels of SPARC, IL-10 and IL-13 over the 24h of blood collection. IL-6 was the only myokine significantly increased in plasma immediately after the exercise bout, and the absolute increase relative to baseline was similar in both groups. At all time-points (basal levels and levels in response to the exercise bout), SPARC and IL-8 levels in plasma were lower in the obese group, while IL-13 levels were higher in obese women in comparison to non-obese subjects. Quantification of the AUC indicated a trend towards decreased overall IL-8 ($p=0.087$) and SPARC ($p=0.073$) in the obese group. Finally, a significant interaction between obesity status and the variation in FGF21 levels following the exercise bout was detected, suggesting the rise in levels of this myokine in plasma after acute exercise was earlier in the participants with obesity and they remained elevated after 24h rather than returning to basal as in the non-obese group.

## Discussion

This study focused on the comparison in plasma myokine levels in women with obesity vs. women without obesity after a bout of moderate-intensity continuous cycling. Plasma myokines were assessed over a 24h time-course following the acute exercise to account for the different timing of secretion of individual peptides. Most of the myokines measured were regulated by acute exercise (FGF21, IL-6, IL-8, IL-15 and IL-18), and some were released at different levels in plasma over the 24h in the group of women with obesity in comparison to the non-obese group (SPARC, IL-8 and IL-13). The regulation of plasma FGF21 following acute exercise was different in the non-obese group in comparison to the obese group.

**IL-6.** Regarding IL-6, the magnitude and the time-course of the increase in plasma IL-6 following the exercise bout was similar to a previous study in which normal-weight and obese subjects had completed 30 minutes of moderate-intensity aerobic exercise (Slusher et al. 2015). In muscle biopsies from healthy individuals taken at different time-points following a running bout, IL-6 mRNA expression was found to be induced immediately by exercise (Louis et al. 2007). These results align with the variations in circulating IL-6 levels that we observed over the 24h following the acute exercise bout completed by our sedentary participants. As reviewed by Lombardi et al., acute increases in IL-6 secretion are better detected in plasma than serum, further highlighting the relevance of our results (Lombardi et al. 2017).

**IL-8.** No increase in plasma IL-8 was detected after the cycling bout, although others found increased muscle IL-8 expression after a running exercise (Louis, Raue, Yang, Jemiolo and Trappe 2007). This suggests that the increased expression of IL-8 during muscle contraction might result in the release of the protein within the muscle *interstitium*. These results support the hypothesis of Nielsen and colleagues regarding the potential autocrine role of IL-8 in muscle metabolic adaptations to physical activity (Nielsen and Pedersen 2007). On the other hand, IL-8 levels are

higher in serum than plasma (Lombardi, Sansoni and Banfi 2017). Perhaps differences in circulating IL-8 levels after the exercise bout would have been more significant in serum than in plasma, as this myokine seems to be released primarily in that fraction of the blood. Circulating resting and exercise-induced IL-8 was reduced in participants from the obese group, which could potentially be explained by their body composition, as women in the non-obese group had significantly less total lean and fat mass. Since myokines were measured solely in the circulation, we cannot hypothesize on an effect of either tissue to explain this difference between the study groups.

**IL-10.** To our knowledge, no studies have been published on the effect of exercise on circulating levels of IL-10 in metabolically healthy subjects. On the other hand, in support of our findings, others found no effect of acute exercise on plasma IL-10 levels in patients with T2D (Korb et al. 2018). Since this myokine has been shown to have a positive effect on skeletal muscle metabolism (Dagdeviren et al. 2016, Hong et al. 2009), perhaps its mechanism of action in response to exercise is autocrine and unrelated to circulating concentrations. This hypothesis would support our findings, as well as those of others regarding the lack of response to an acute bout of exercise in circulating IL-10. A more molecular analysis of the signaling mechanisms in muscle *per se* would be required to elucidate the effects of exercise on muscle IL-10 secretion and/or expression.

**IL-13.** Concerning IL-13, plasma levels were higher in the obese in comparison to the non-obese group at rest and in response to the acute bout of exercise. Others have shown that serum and muscle IL-13 is reduced in non-obese patients with T2D, a common comorbidity of obesity (Jiang, Franck, Egan, Sjogren, Katayama, Duque-Guimaraes, Arner, Zierath and Krook 2013). Further investigations would be required to assess how obesity and/or T2D affect circulating IL-13. Our results for plasma IL-13 in response to acute exercise showed great variability, preventing us to

draw any clear conclusion. In many samples, IL-13 was undetected, which affected the power to detect any significant differences between groups or in response to exercise.

**IL-15.** Our results demonstrated that IL-15 circulating levels were not immediately increased after the bout of cycling, although this myokine was previously found to be higher in plasma after an acute bout of cycling exercise (Sabaratnam, Pedersen, Kristensen, Handberg, Wojtaszewski and Hojlund 2018). In their study, plasma IL-15 levels returned to lower levels than baseline 3h into recovery, while we show a steady increase as far out as 12h. These discrepancies could be explained by the fact that our participants were women, while theirs were men. On another note, an acute running bout in healthy participants induced a gradual increase in muscle IL-15 mRNA expression over the 24h following exercise, aligning with our results (Louis, Raue, Yang, Jemiolo and Trappe 2007). Furthermore, IL-15 can be found in circulation in its free form or complexed to a soluble form of the alpha subunit of the IL-15 receptor (sIL-15Ralpha), which may alter IL-15 activity depending on the target cells as discussed in (Nadeau and Aguer 2019). The kit used in the study mentioned previously shows around 21% cross-reactivity with the complexed form of IL-15 according to the manufacturer (Human IL-15 Quantikine ELISA Kit, R&D Systems Inc., MN, USA), while the one we used (MSD) showed average reactivity at physiologically relevant doses (1.1-17.2 pg/ml) of about 38% (Supplementary figure 1). Since the half-life of free IL-15 is relatively short (about 30 minutes) and increases when IL-15 is complexed to sIL-15R$\alpha$ (20-25h), it is likely that the increase we observed after acute exercise lasting into the recovery period was due to the recognition of the complex, as discussed in (Nadeau and Aguer 2019). Further studies would be required to better understand the mechanisms of free IL-15 and IL-15/sIL-15Ralpha complex secretion in response to exercise. Moreover, resting plasma IL-15 levels were found to be lower in physically active individuals in comparison to sedentary subjects and even higher in

sedentary obese patients (Perez-Lopez, Valades, Martinez, Blanco, Bujan and Garcia-Honduvilla 2018). Therefore, we anticipated that the group of women with obesity would show higher plasma IL-15, but we found no significant differences between the two groups. Since Perez-Lopez and colleagues used the kit mentioned previously to detect IL-15, we again speculate that the differences in our results stem from the detection of the complexed form of IL-15 in our assay. Finally, the matrix does not affect IL-15 recovery when measuring this myokine in the circulation, which suggests the results would have been similar if serum samples had been analyzed (Lombardi, Sansoni and Banfi 2017).

**SPARC.** Circulating SPARC levels were unaffected immediately after an acute exercise bout, although others found an increase in serum SPARC in rats after acute exercise that mirrored increased muscle protein content (Matsuo et al. 2017). The same group also found a transient increase in serum SPARC in humans after a bout of high-intensity interval exercise (HIIE) or moderate-intensity continuous exercise (MICE). Data relating to pre-analytical parameters for the quantification of SPARC in the circulation are lacking; it is possible, however, that variations in SPARC levels after exercise are better detected in serum than plasma. Nevertheless, others showed that a single 20 seconds 'all-out' cycling sprint induced a significant rise in serum SPARC, but that correcting circulating SPARC levels with the exercise-induced changes in plasma volume negated this effect (Songsorn et al. 2017). This finding highlights the importance of measuring hematocrit and hemoglobin levels pre- and post-exercise to verify that the potential exercise-induced alterations in circulating protein concentrations are not due to the changes in plasma volume after exercise (Alis et al. 2015, Dill and Costill 1974).

**FGF21.** FGF21 is primarily expressed and produced by the liver (Nishimura et al. 2000), but its expression is also induced in skeletal muscle during exercise and serum FGF21 increases following

an aerobic exercise bout (Tanimura et al. 2016). We observed no significant increase in plasma FGF21 after the cycling bout in either groups, which confirms the findings of others regarding changes in plasma or serum FGF21 after exercise in patients with obesity, but contradicts the results obtained in healthy participants (Sabaratnam, Pedersen, Kristensen, Handberg, Wojtaszewski and Hojlund 2018, Slusher, Whitehurst, Zoeller, Mock, Maharaj and Huang 2015). In normal weight (BMI 18.5-24.9 kg/m$^2$) men and women, FGF21 was found to be acutely increased in plasma after exercise; however, since our sedentary group had an average BMI classified as overweight (25-29.9 kg/m$^2$), perhaps the response in FGF21 secretion was altered similarly as in patients with obesity. Of note, the average circulating FGF21 levels detected in our groups were slightly elevated compared to values from previous studies. Hansen *et al.* compared the effect of exercise on FGF21 release from the liver in comparison to leg muscle and found no significant release of this protein in the circulation from skeletal muscle, whereas the splanchnic bed secreted up to four-fold more FGF21 after acute exercise (Hansen et al. 2015). These findings confirm the hypothesis that skeletal muscle is not the main source of circulating FGF21, even during exercise.

**Short-comings and Limitations.** Our study design did not allow for measurement of myokine secretion directly from skeletal muscle, as similar peptides can be secreted by other tissues (e.g. adipose tissue, liver, brain, etc.) (Garneau and Aguer 2019, Weigert et al. 2019). Therefore, we cannot say for certain that all of the measured peptides originated from skeletal muscle in response to exercise. However, we found no correlations between any of the myokines and fat mass or BMI (data not shown), suggesting that adipose tissue is likely not responsible for any of the observed effects of acute exercise on variations in circulating myokines. Nevertheless, it is possible that other organs, such as the liver, might have contributed to the changes in circulating peptides levels

following the cycling bout, as discussed regarding FGF21. A solution to this problem is the use of skeletal muscle biopsies to quantify myokine secretion directly from muscle (Garneau and Aguer 2019). However, myokines measured in skeletal muscle cannot be assumed to originate solely from muscle secretion, as the peptides could be secreted by other tissues to act on skeletal muscle during exercise. Also, increased levels of a peptide in muscle after exercise does not necessarily translate into its release in the circulation.

Although some of the myokines measured are more stable and/or better detected in plasma, others are ideally quantified in serum. Therefore, changes in myokine secretion in the circulation might go undetected by analyses performed solely in plasma samples. Another limitation of our analyses consists in the single measurement of every sample, preventing us to calculate intra- or inter-assay coefficients of variation for all myokines. In addition, exercise modalities might be great contributors to the outcome on muscle signaling. The combination of aerobic and resistance exercises confers more significant changes in glucose homeostasis and muscle substrate metabolism in patients with T2D and is therefore of great interest regarding muscle signaling adaptations to exercise (Church et al. 2010, Sparks et al. 2013). Similarly, interval exercise of elevated intensity such as Tabata exercises or sprint intervals has been shown to stimulate myokine release acutely in the circulation (Harnish and Sabo 2016). Consequently, the plasma concentrations of myokines in the 24h following an exercise bout might evolve differently as a function of the exercise modality performed.

Another confounding effect in our study design that could have affected the measurement of certain myokines that are also cytokines (the interleukins) is the use of a venous catheter for blood collection. This method was chosen as it is less invasive and stressful than repetitive venipuncture for subjects undergoing eight blood draws over 24h. However, others have shown that this method

of blood sampling could induce local inflammation and affect levels of circulating cytokines to a greater extent than venipuncture (Dixon et al. 2009). Also, it has been shown that differences in plasma volume can affect the analysis of exercise-induced circulating myokine variations (Songsorn, Ruffino and Vollaard 2017). Since we did not have values for hematocrit and hemoglobin for the time-points following the cycling bout, we were unable to measure plasma volume variations as a function of time during the recovery period and therefore could not correct circulating myokine levels to variations in blood composition (Dill and Costill 1974). As we did not detect an increase of all the myokines measured in plasma following acute exercise, it is likely, nonetheless, that differences in plasma volume did not cause erroneous conclusions regarding exercise-induced myokines. Moreover, inter-individual variability is a major source of concern in observing the muscle signaling adaptations to acute or chronic exercise (He et al. 2018), and this could explain the impossibility to find an effect of acute exercise for certain peptides that we measured in plasma. Likewise, since our sample size was very small, some significant effects of exercise might have been undetected. This factor is the greatest limitation of our study. Regardless, we were able to find interesting effects of both acute exercise and obesity status on circulating target myokines amongst our study groups. Another limitation of our study is the fact that only women were represented, but this feature can also be considered positive, as women are generally underrepresented in the literature regarding exercise metabolism and muscle signaling.

**Conclusion.** Our findings relating to the time course of plasma myokine levels following acute exercise depending on BMI classification (obese or non-obese) shed light on the mechanisms of endocrine signaling after physical activity. We found a regulation of IL-6, IL-8, IL-15, IL-18 and FGF21 in plasma by exercise, while obesity status increased IL-13 and decreased IL-8 and SPARC in the circulation. The next step would be to assess the effects of acute exercise on muscle signaling

directly in these two groups, for example, by measuring myokine secretion from muscle biopsies harvested before and after the exercise bout. In this sense, it would be possible to compare signaling adaptations in participants with or without obesity in the circulation with the ones observed locally in skeletal muscle and potential correlations or distinctions between myokine regulation at both levels could be made as a function of body composition (*i.e.* BMI).

## Conflict of Interest and Ethics

The authors (SRS, SAP and LMS) declare that this study received funding from Takeda Pharmaceuticals Inc. None of the funding sources played a role in the collection, analysis, or interpretation of the data or in the decision to submit the manuscript for publication. All authors declare no conflict of interest.

The Institutional Review Board at AdventHealth Orlando approved this research for ethical standards, scientific merit, and regulatory compliance. The Office of Research Administration (ORA) provided support and oversight to ensure the integrity of this research at AdventHealth Orlando. ClinicalTrials.org Identifier: NCT01911091.

## Author Contributions

All samples were provided by the laboratories of LMS and SRS. Sample analyses, data collection and analyses were performed by CA and LG at EEM's laboratory facilities. All authors contributed to the review of the manuscript.

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## Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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## Figure

## Figure 1

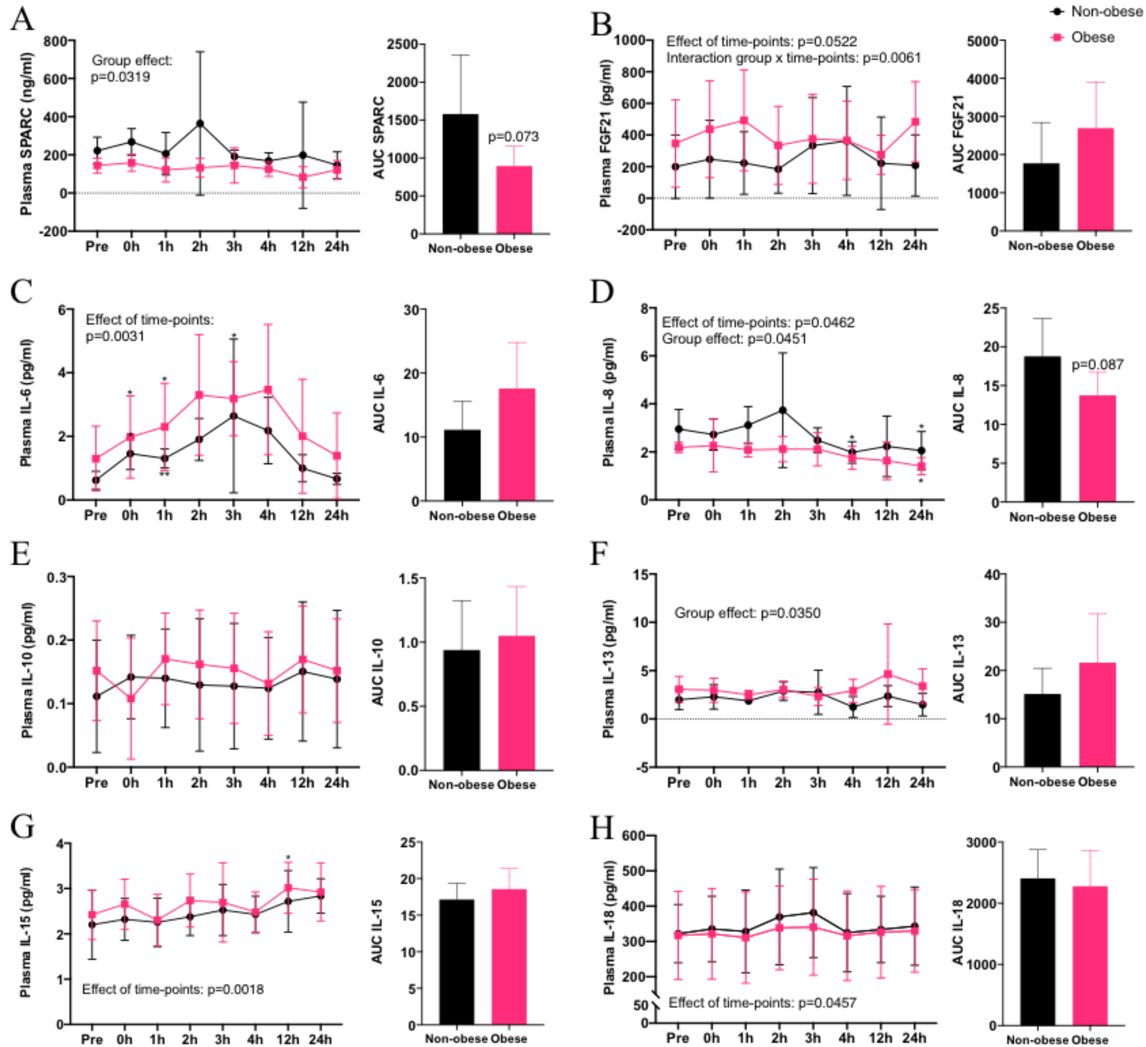



*Figure 1. Myokines in circulation after an acute bout of exercise in women with or without obesity. Time course of concentrations of myokines in plasma of non-obese and obese women following 60 minutes of cycling at 60% VO2peak at 8 different time-points (pre-, immediately after (0h), 1h, 2h, 3h, 4h, 12h and 24h into recovery) and area under the curve (AUC) of all time-points. (A) SPARC, (B) FGF21, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) IL-15 and (H) IL-18. Non-obese group, n=5 (black circles) and obese group, n=6 (pink squares). *p<0.05, **p<0.01 in comparison to pre-exercise (pre), effect of time-points relates to variations after the exercise bout and group effect relates to BMI classifications.*

## Table

*Table 1. Participant characteristics of women having completed a single, acute bout of moderate intensity continuous exercise.*

| Group | n | Age (years) | BMI (kg/m$^2$) | Fat mass (kg) | Lean mass (kg) | Glucose (mmol/L) | Insulin (μIU/mL) | HOMA-IR | HbA1c (%) | $VO_2$max (mL/min) |
|---|---|---|---|---|---|---|---|---|---|---|
| Non-obese | 5 | 28.80 ± 8.41 | 27.38 ± 1.40 | 27.69 ± 4.95 | 38.16 ± 4.54 | 5.01 ± 0.43 | 14.04 ± 7.80 | 3.21 ± 1.92 | - | 1606 ± 305 |
| Obese | 6 | 30.00 ± 6.93 | 35.43 ± 5.44$^{\dagger}$ | 45.93 ± 12.43$^{\dagger}$ | 50.22 ± 2.84$^{\dagger\dagger}$ | 5.02 ± 0.32 | 14.48 ± 4.96 | 3.28 ± 1.30 | 5.73 ± 0.33 | 1817 ± 282 |

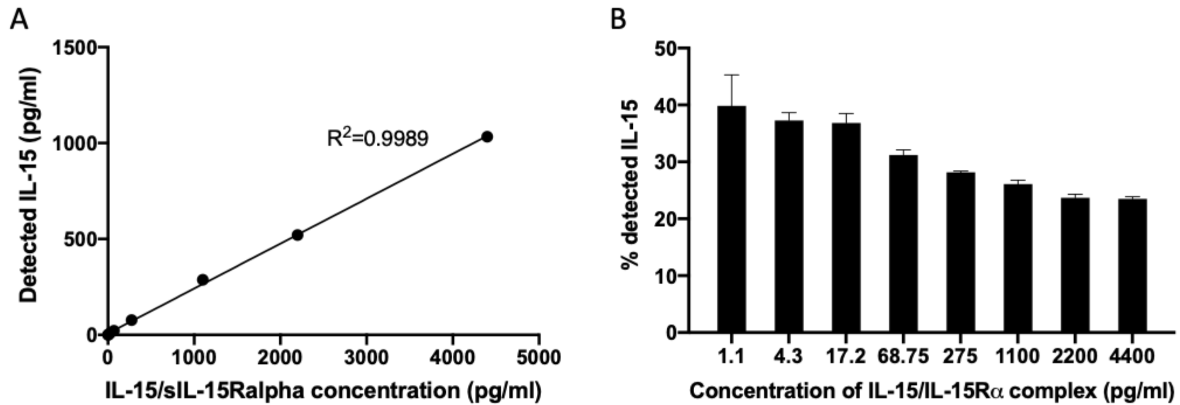
Data are means ± SD. $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ in comparison to non-obese group.

## Supplementary Material

**Materials and Methods.**

Direct links to datasheets of all antibodies used:

- IL-6: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-6.pdf
- IL-8: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-8.pdf
- IL-10: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-10.pdf
- IL-13: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-13.pdf
- IL-15: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-15.pdf
- IL-18: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20u-plex%20human%20il-18.pdf
- FGF21: https://www.mesoscale.com/~/media/files/data%20sheets/ds-u-plex-human-fgf-21.pdf
- SPARC: https://www.mesoscale.com/~/media/files/data%20sheets/ds%20r-plex%20human%20osteonectin.pdf




*Supplementary figure 1. Cross-reactivity of IL-15 antibody to the complex IL-15/IL-15Rα. Different concentrations of recombinant IL-15/IL-15Rα (R&D System) were assayed in a multiplex plate with IL-15 anti-body from MSD. (A) Measured free IL-15 (pg/ml) as a function of loaded IL-15/sIL-15Ralpha concentration (pg/ml). (B) Detected IL-15 concentration (pg/ml) was divided by loaded concentration of the complex to obtain a percentage of cross-reactivity for the assay. Data is presented ± SD, n=2 for all concentrations, except 2200 pg/ml n=3.*

# Chapter 3

# Exercise Training Reduces Circulating Cytokines in Male Patients with Coronary Artery Disease and Type 2 Diabetes: a Pilot Study

This study's objective was to determine whether different exercise training modalities (*i.e.* MICT and HIIT) could impact peripheral myokine regulation differently, as their effects on glucose homeostasis have been shown to differ in people living with T2D. We also wanted to determine if alterations in myokine secretion would differ between individuals with or without T2D. The two different exercise training interventions were based on cardiac rehabilitation programs and performed by a population of patients with CAD with or without T2D. The randomized clinical trial (NCT02765568) was performed at the University of Ottawa Heart Institute by Dr. Jennifer Reed's team. Blood samples were shared with our team for the analysis of circulating cytokine levels pre- and post-intervention. Although the peptides quantified in the plasma samples are all cytokines (*i.e.* TNF-α, IL-1β and CRP), some of them are also myokines (*i.e.* FGF21, SPARC, IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18), as muscle cells are capable of secreting them. We were able to observe the effects of 12 weeks of either MICT or HIIT on the regulation of these cytokines in circulation, therefore inferring the chronic anti-inflammatory potential of both protocols. These findings are of interest considering chronic inflammation is a central factor in the development of CAD, an important contributor to the co-morbidity with T2D., and HIIT shows great potential in helping patients with T2D for the management of their symptoms.

This study was published in the journal Physiological Reports.

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**Exercise training reduces circulating cytokines in male patients with coronary artery disease and type 2 diabetes: a pilot study**

**Abbreviated title:** Exercise training, cytokines and cardiometabolism

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## Abstract

*Context.* Low-grade inflammation is central to coronary artery disease (CAD) and type 2 diabetes (T2D) and is reduced by exercise training.

*Objective.* Comparison of anti-inflammatory potential of moderate-to-vigorous intensity continuous (MICT) and high-intensity interval (HIIT) trainings in patients with CAD with or without T2D.

*Design and setting.* Secondary analysis of registered randomized clinical trial (NCT02765568).

*Patients.* Male patients with CAD were randomly assigned to either MICT or HIIT, with sub-groups divided according to T2D status (non-T2D-HIIT n=14 and non-T2D-MICT n=13; T2D-HIIT n=6 and T2D-MICT n=5).

*Intervention.* A 12-week cardiovascular rehabilitation program consisting of either MICT or HIIT (twice weekly sessions) and circulating cytokines measured pre- and post-training as inflammatory markers.

*Results*. The co-occurrence of CAD and T2D was associated with increased plasma IL-8 ($p=0.0331$). There was an interaction between T2D and the effect of the training interventions on plasma FGF21 ($p=0.0368$) and IL-6 ($p=0.0385$), which were further reduced in the T2D groups. An interaction between T2D, training modalities, and the effect of time ($p=0.0415$) was detected for SPARC, with HIIT increasing circulating concentrations in the control group, while lowering them in the T2D group, and the inverse occurring with MICT. The interventions also reduced plasma FGF21 ($p=0.0030$), IL-6 ($p=0.0101$), IL-8 ($p=0.0087$), IL-10 ($p<0.0001$), and IL-18 ($p=0.0009$) irrespective of training modality or T2D status.

*Conclusion*. HIIT and MICT resulted in similar reductions in circulating cytokines known to be increased in the context of low-grade inflammation in CAD patients, an effect more pronounced in patients with T2D for FGF21 and IL-6.

**Keywords:** Type 2 diabetes, coronary artery disease, exercise, interleukins, cytokines

# 1. Introduction

Coronary artery disease (CAD) is the most common form of heart disease. Following coronary artery revascularization, patients with CAD are referred to cardiovascular rehabilitation programs to improve patients' physical and mental health (1-3). Conventional cardiovascular rehabilitation programs include moderate-to-vigorous intensity continuous training (MICT) to assist patients in attaining weekly exercise recommendations (*i.e.,* 150 minutes of moderate-to-vigorous-intensity exercise per week in combination with light physical activity (4), with at least two sessions per week incorporating exercises to strengthen muscles and bones (resistance training) (5)) and improving their cardiovascular health. Type 2 diabetes (T2D) is a non-communicable chronic disease often associated with a sedentary lifestyle (prolonged sitting time) and or lack of physical activity, and excess body weight (6-8). T2D is also strongly associated with the development and progression of CAD (9). In a study evaluating the long-term outcomes of coronary artery bypass graft surgery (CABG) in patients with CAD, the presence of T2D resulted in increased morbidity and mortality over the first 10 years following the surgery, and this increase was exacerbated in patients taking anti-hyperglycemic medications and insulin (10).

Individuals who suffer from T2D often experience difficulties in engaging in regular exercise due to physical discomforts associated with their disease (*e.g.,* fear of injury, pain related to movement, hypoglycemia, tiredness, etc.), as well as psychological factors affecting their participation in exercise (*e.g.,* depression, shame, lack of motivation, laziness, fear of the perception of others, etc.) (11). Regardless of existing pathologies, lack of time is frequently

cited as a barrier to regular exercise participation and meeting current World Health Organization activity guidelines (*i.e.*, at least 150 minutes/week of moderate to vigorous aerobic exercise) (12). Many researchers have, thus, explored high-intensity interval training (HIIT) as an alternative to traditional MICT in achieving similar or superior physical and mental health improvements in less time. HIIT has shown promise for the management of T2D as it was shown to induce similar or greater improvements in glucose homeostasis when compared to MICT, even with 45% reduced training volume (13).

Atherosclerosis is driven by the accumulation of cholesterol within the arterial wall and chronic non-resolving inflammation (14), which is also an important feature of metabolic syndrome, insulin resistance, and T2D (15). The state of chronic inflammation induced by imbalances between the regulation of pro-inflammatory cytokines and chemokines (*e.g.,* interleukin (IL)-1b, tumour necrosis factor (TNF)-a, IL-6 and IL-8) and anti-inflammatory cytokines (*e.g.,* IL-10 and IL-13) results in impaired glucose and lipid metabolism, tissue dysfunction, and contributes to residual inflammatory risk in cardiovascular death (16). This underscores the importance of reducing chronic inflammation in patients with CAD with or without T2D. In a recent meta-analysis examining the effect of exercise interventions (HIIT ~27% of all studies; MICT ~76%; MICT or HIIT in combination with resistance training ~22%) on circulating inflammatory markers in patients with CAD, neither significant reductions in TNF-a, IL-6 or IL-8 concentrations , nor increases in IL-10 were found; however, a reduction in C-reactive protein (CRP) suggested diminished acute phase inflammation without changes in chronic inflammatory markers (17). Direct comparisons of the anti-inflammatory potential of HIIT and

MICT are scarce in the literature and both short- (2 weeks) (18, 19) and longer-term (10-12 weeks) interventions (20, 21) resulted in no changes in circulating inflammatory markers in various populations (healthy individuals, patients with obesity and/or T2D). The exception is one 8-week protocol of HIIT which led to increased serum CRP and IL-6, while no significant change was measured with the MICT protocol in the same cohort of overweight or obese participants (22). Because most comparisons between exercise modalities are drawn through meta-analyses with few direct comparisons of HIIT and MICT interventions in clinical populations, the effects of such training on reducing chronic inflammation remains unknown.

The current study assessed the impact of 12 weeks of HIIT- or MICT-based cardiovascular rehabilitation on plasma cytokine concentrations in patients with CAD with or without T2D. We examined eleven target cytokines (IL-1b, TNF-a, CRP, Secreted Protein Acidic Rich in Cystein (SPARC), Fibroblast Growth Factor 21 (FGF21), IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18) known to be altered in the presence of obesity and/or T2D, and to be regulated by acute and/or chronic exercise (23). It was hypothesized that HIIT and MICT modalities would induce a more significant reduction in circulating cytokines concentrations in the patients with CAD and T2D, as these patients are affected with more pronounced chronic inflammation. As HIIT has been shown to further improve glucose homeostasis than MICT in patients with T2D, we also sought to determine if this training modality would be more effective in reducing inflammatory markers in these patients.

## 2. Results

*2.1. Impact of the training interventions on participants' characteristics and functional capacity*

The number of participants in each group were as follows: n=14 for non-T2D-HIIT, n=13 for non-T2D-MICT, n=5 for T2D-HIIT and n=6 for T2D-MICT. On average, participants were classified as obese (average BMI: 30.4 kg/m$^2$) and were normotensive (average systolic/diastolic blood pressure: 123/79 mmHg) due to medical management. The list of medications taken by the participants are reported in Appendix A.

The anthropometric and metabolic characteristics of the study participants pre- and post-training interventions are presented in Table 1. No significant differences were found in any group at baseline or in response to the training interventions for age, BMI, waist circumference, body fat %, resting systolic or diastolic blood pressure. There was a significant interaction between the effect of time and training modality on changes in systolic blood pressure ($p=0.0455$), such that values increased in the HIIT groups, but decreased in the MICT groups following the training interventions. We found no effect of time or training modality on fasting blood glucose concentrations, although these were significantly increased in the groups of patients with than without T2D ($p=0.0015$). As expected, the HbA1c values of both groups with T2D pre- and post-training interventions were higher than those of participants without T2D ($p<0.0001$), with no significant effect of the training interventions. Following the interventions, regardless of training modality or T2D status, participants' resting heart rate was

reduced compared to baseline measurements (p=0.0129) and the distance achieved during the 6MWT increased (p<0.0001).

*2.2. Regulation of plasma cytokine levels before and following the training interventions*

All cytokines measured were detected in the totality of the samples (HIIT non-T2D n=14; MICT non-T2D n=13; HIIT T2D n=5; MICT T2D n=6) except for IL-13, which was undetected in roughly half of the participants' samples irrespective of group. The process of identification of outliers resulted in the exclusion of certain data points for IL-1b, TNF-a, CRP, FGF21, IL-6, IL-8, IL-10 IL-15 as well as IL-18, and all the outliers were found in the non-T2D groups.

We found no effect of time (before and after training interventions consisting of HIIT or MICT), nor of the co-morbidity with CAD and T2D on plasma IL-1b, TNF-a and CRP concentrations (Fig. 1 A-C). Although no effect of time or type of exercise was detected for SPARC, there was a significant interaction between the presence of T2D in the participants and the plasma SPARC levels as a function of training modality (p=0.0124), as well as between T2D, the training modalities and the effect of time (p=0.0415) (Fig. 2 A). This is portrayed by varying plasma concentrations of SPARC at all time-points between the groups. The variations in response to training were also different between groups, with an increase in plasma SPARC concentrations in the HIIT-non-T2D group and a reduction in the HIIT-T2D group, whereas the contrary occurred in the two MICT groups. Furthermore, in response to the 12-week supervised exercise protocols, regardless of the exercise modalities, reductions in resting plasma concentrations of FGF21 (p=0.0030), IL-6 (p=0.0101), IL-8 (p=0.0087), IL-10

($p<0.0001$), and IL-18 ($p=0.0009$) were observed (Fig. 2 B-E and H). The reductions in plasma FGF21 in the HIIT-T2D group ($p=0.0499$) post-intervention reached significance in comparison to pre-intervention concentrations (Fig. 2 B). An interaction between the co-occurrence of T2D and CAD in the patients and the changes in plasma concentrations of FGF21 ($p=0.0368$) and IL-6 ($p=0.0385$) in response to training was also detected independently of training modality. The reductions in these cytokines were more pronounced in the groups with T2D. No effects of either HIIT or MICT interventions were detected on resting IL-13 and IL-15 plasma concentrations (Fig. 2 F and G). At all time-points, plasma IL-8 concentrations were significantly elevated in the T2D groups in comparison to non-T2D ($p=0.0331$) (Fig. 2 D). No significant effect of T2D was found for any of the other cytokines.

## 3. Discussion

Chronic inflammation is a hallmark of diseases such as CAD and T2D, as elevated concentrations of specific cytokines and other signaling factors are found in the circulation in these patients and often correlate with the severity of their disease (27, 28). Regular exercise has conversely been shown to reduce the risk of cardiovascular events through it's influence on improving metabolic parameters (*e.g,*. HbA1c, blood lipid profile, etc.) in patients with CAD with or without T2D (29). The objective of our study was to compare the anti-inflammatory potential of traditional (*i.e.,* MICT) and alternative (*i.e.,* HIIT) exercise interventions on the regulation of circulating cytokine concentrations in patients with CAD with or without T2D. We specifically targeted cytokines as markers of chronic inflammation because most of these signaling peptides are increased in the circulation of patients with metabolic syndrome and/or T2D (15). Consistent with our original hypothesis, the plasma concentrations of certain cytokines (*i.e.,* FGF21 and IL-6) were further reduced following the training interventions in the groups of patients with T2D. Further, both training modalities yielded significant decreases in plasma cytokine concentrations (FGF21, IL-6, IL-8, IL-10 and IL-18), suggesting that the 12-week exercise interventions were sufficient to reduce chronic inflammation in these patients.

### 3*.1. The effect of 12 weeks of exercise training on circulating cytokine concentrations*

Surprisingly, we found no significant effect of any of the training interventions on plasma concentrations of IL-1b, TNF-a and CRP in the patients with CAD with or without T2D. The

lack of changes in plasma TNF-a concentrations following an exercise training intervention in patients with CAD is consistent with the finding of others following a 6-month HIIT-based intervention (30). However, a 12-week MICT-based intervention in CAD patients was found to result in a significant reduction in circulating CRP and IL-1 concentrations, contrary to what we observed in our patient population (31). These differences in findings could be due to several factors, including different assays to measure the cytokines and the presence of angina in their patient population. Regardless, a recent meta-analysis by Hejazi *et al.*, showed a consensus in the reductive effect of aerobic exercise training interventions on circulating TNF-a and CRP concentrations in patients with CAD (32). Interestingly, their analysis of variations in circulating TNF-a and CRP concentrations in response to aerobic training interventions showed that patients with a BMI ≥ 30 kg/m$^2$ did not experience significant reductions in either cytokine, while those with BMI 25.0–29.9 kg/m$^2$ did not show reduced circulating CRP concentrations following the interventions. On the contrary, a reduction in circulating CRP concentrations was achieved with exercise training in patients with CAD and a BMI < 25kg/m$^2$. Consequently, it is possible that the absence of significant changes in circulating levels of these cytokines known to be influenced by exercise levels in patients with CAD could be explained by the demographics of our patient population, since 19 (50%) participants at baseline had a BMI 25.0–29.9 kg/m$^2$, 16 (42%) had a BMI ≥ 30 kg/m$^2$, and 3 (8%) had a BMI 20.0–24.9 kg/m$^2$. A reduction in circulating FGF21 concentrations (-253.8 pg/mL) was observed across all groups following the 12 weeks of exercise training. Kruse *et al.* found that 10 weeks of MICT in patients with T2D did not affect serum concentrations of FGF21 (33). This

discrepancy may be due to the medium analyzed (serum versus plasma; as the medium in which cytokines are measured can affect the outcome of interventions on the variation of their circulating concentrations (34)) or the shorter duration of the aerobic exercise bouts in their study (20-35 minutes). Similarly, across all groups, plasma IL-6 concentrations (-0.250 pg/mL) were reduced following the exercise intervention. This finding is consistent with those of Sabouri *et al.* in a cohort of patients with T2D that includes both sexes following a 12-week (three sessions per week) HIIT intervention (35) and in patients with CAD following a 12-week MICT-based intervention (31). A reduction in plasma IL-8 concentrations was also detected across all of our groups (-0.693 pg/mL). The reductions in both circulating IL-6 and IL-8 concentrations are similar to the results of Munk *et al.*, who measured a reduction in both cytokines in the plasma of patients with CAD following 6 months of HIIT with a similar protocol performed either on a treadmill or cycle ergometer (3 times per week with 4x4 minutes bouts interspersed with 3 minutes of active rest and some strength and stretching exercises) (30). Plasma IL-15 concentrations were unchanged following the training interventions (-0.534 pg/mL) irrespective of T2D status (p=0.0562). These findings contrast those of Perez-Lopez *et al.* in healthy and obese individuals, for which habitual physical activity was associated with lower serum concentrations of IL-15. On the contrary, we expected a reduction in plasma IL-15 concentrations (36). It is possible that if our interventions were performed in a larger group of participants, specifically patients with T2D co-morbidity, it may have allowed for the detection of significant reductions, as our p-value was close to significance. Also, we demonstrated that 12 weeks of HIIT or MICT resulted in a reduction in plasma IL-18

concentrations (-82.19 pg/mL) with no differences between groups regarding T2D status. A study from Zaidi et al. revealed no effect of a 12-month aerobic exercise intervention including HIIT-based sessions was detected on serum IL-18 concentrations in a cohort of patients with CAD and T2D composed mostly of men (37). They also observed no reduction in adipose tissue or leukocyte IL-18 expression following the training intervention. We did not observe a significant reduction in total body fat percentage following our exercise training interventions. This suggests that the observed reduction in plasma IL-18 concentrations in response to the 12-week exercise interventions in the patients with CAD with or without T2D is likely not due to reduced secretion of this cytokine from adipose tissue in accordance with the findings of Zaidi *et al.* discussed above.

Although we observed a reduction of the anti-inflammatory cytokine IL-10 (-0.155 pg/mL) in the plasma of all groups following training, Munk *et al.* detected an increase in circulating IL-10 after 6 months of HIIT (30) and Goldhammer *et al.* showed that 12 weeks of MICT in patients with CAD also increased plasma concentrations of this cytokine (31). Notably, the plasma concentrations measured in these studies were approximately 3 to 5-fold higher than those of our cohort. This could be explained by the duration of the training protocol in the case of the Munk *et al.* study (*i.e.*, 6 months vs. 12 weeks), the different timing of the blood sample in relation to procedures and intervention, as well as the medical conditions of the patients (*i.e.,* angina vs. CAD without angina), all potentially confounding the comparison between studies. Also, although the kit used to measure IL-10 by Munk *et al.* was not described in their methods section, the one used by Goldhammer *et al.* was a high-sensitivity quantitative enzyme

sandwich immunoassay from a different manufacturer and based on different technology for detection (*i.e.*, colorimetric vs. sulfo-tag). Nonetheless, it would seem counterintuitive that an anti-inflammatory cytokine or cytokines that have been shown to have a dual role in inflammatory pathways (*i.e.*, IL-6 and IL-18) be diminished in the circulation of patients following a training intervention. An increase in anti-inflammatory cytokines would be expected. The reduction we observed could be the counterbalance of lower concentrations of pro-inflammatory cytokines following the training interventions in the patients with CAD.

*3.2. HIIT and MICT have a similar effect on plasma cytokine concentrations*

Few studies have compared the effects of HIIT- and MICT-based exercise interventions on circulating cytokines in clinical populations. In a 12-month randomized controlled trial of combined aerobic and strength training (3 sessions per week) including only patients with T2D, greater reductions in plasma concentrations of IL-6 were detected following MICT than HIIT in comparison to pre-intervention values (38). The interventions employed by Magalhaes *et al.* included three sessions of cycling per week matched for energy expenditure between modalities, which gradually increased in intensity up to 40-60% heart rate reserve (HRR) in the MICT group, and up to 90% HRR for 1-minute bouts interspersed with 1 minute of active rest in the HIIT group for a duration matching the prescribed energy expenditure. In the current study, we showed that both training interventions yielded similar reductions in plasma IL-6 over 12 weeks. The differences between our observations in IL-6 concentrations in the plasma according to the training modalities could arise from the different types of HIIT protocols employed in both cases, as the intervals in our study were of longer duration and at a lower

intensity those of Magalhaes *et al.*. Of note, the measured values for circulating IL-6 in the aforementioned study were approximately 10-fold higher than ours and most of the data in the literature and their study groups were comprised of men and women in approximately even proportions, while only men were included in our study.

*3.3. The impact of T2D co-morbidity in patients with CAD and their peripheral cytokine levels*

We found no differences in plasma SPARC concentrations between patients with or without T2D, while Wang *et al.* observed a correlation between serum SPARC concentrations and the homeostatic model assessment of insulin resistance (HOMA-IR) index in patients with CAD (39). This discrepancy between the two findings could be due to the medium in which SPARC was measured, as we quantified the cytokine in plasma rather than serum. In patients with CAD, serum FGF21 concentrations are elevated in comparison to healthy subjects and the effect is more pronounced with additional metabolic disorders, such as T2D (40). Our findings are not consistent with the literature, as we noted no effect of T2D in increasing plasma FGF21 concentrations compared to patients with CAD alone. On the other hand, there was an additive effect of T2D to the reduction in plasma FGF21 in response to training, which was more pronounced in the patients with T2D. The same interaction between T2D status and the effect of time was found for variations in circulating IL-6 concentrations. This confirms our research hypothesis regarding these two cytokines as inflammatory markers that can be further reduced by training interventions in patients with both CAD and T2D, rather than CAD alone. In our cohort of patients with CAD, no effect of the T2D co-morbidity was detected on plasma IL-10 and IL-18 concentrations. In men at risk or diagnosed with CAD, Trøseid *et al.* discovered that

elevated fasting serum glucose or metabolic syndrome did not affect circulating IL-10 concentrations (27). However, they measured higher serum IL-6 and IL-18 in participants with poor glucose homeostasis, which positively correlated with their risk of adverse cardiac events. We did not find increased plasma IL-6 in the groups of patients with T2D, but the reduction in circulating concentrations of this cytokine was greater in patients with T2D in comparison to CAD alone, suggesting an effect of T2D on the response to training. Similarly, Chen *et al.* identified a correlation between circulating IL-18 concentrations and CAD severity (28). In their study population, the occurrence of T2D positively correlated with elevated IL-18 concentrations. Contrastingly, Zaidi *et al.* revealed no relationship between circulating IL-18 concentrations and insulin resistance in patients with both CAD and T2D, but a positive correlation between adipose tissue IL-18 expression and both fasting insulin concentrations and HOMA-IR values (37). This finding suggests that elevated IL-18 concentrations in their patient population might be related to its secretion by adipose tissue. Finally, plasma IL-8 levels were significantly lower in patients with CAD alone in comparison to patients with T2D (p=0.0331), while Trøseid *et al.* demonstrated no effect of metabolic syndrome on IL-8 circulating concentrations (27). Of note, their study population consisted of older men (average age: 70 years old) of which approximately one third were current smokers, and cytokines were measured in serum. Any of these factors could explain this contradiction.

*3.4. Study limitations*

Our study design and findings are novel as little information is available in the literature regarding the concentrations of these cytokines in the circulation of patients with CAD

following a HIIT or MICT exercise intervention. There are several limitations that warrant mention. First, there is the potential confounding effect of medication between the groups. Indeed, one participant in the MICT non-T2D group was taking an oral hypo-glycemic agent at baseline and follow-up. In healthy individuals, oral hypo-glycemic agent such as Metformin may influence concentrations of circulating cytokines in a highly variable manner (41). One participant in the MICT T2D group also began taking insulin between the baseline and follow-up visits. The initiation of insulin medication can lead to lipogenic effects in the first 6 months of treatment, leading to macrophage infiltration in sub-cutaneous adipose tissue and an increase in certain circulating inflammatory factors (*e.g.*, MCP-1, TNF-a and IL-1b) in patients receptive to these effects of insulin treatment (42). Second, there were few (n=5 and 6 respectively for HIIT and MICT) participants in the group of patients with the co-morbidity of T2D. Because of the smaller sample size across all groups, but specifically for participants with T2D, appropriately powered statistical analyses were not performed. The inconclusive results as pertains to IL-13 could, therefore, be explained by low statistical power. A more sensitive assay may have yielded more conclusive results for IL-13 in our patient population. Third, our study did not include any control participants, defined as those not participating in any exercise-based cardiovascular rehabilitation program. Future investigations should consider the inclusion of (1) patients with CAD yet without T2D and (2) patients with CAD and T2D who did not participate in exercise training interventions to control for the effect of time on potential variations in plasma cytokine concentrations. Without this important control group for reference, there is a possibility that the changes we observed in circulating cytokine

concentrations is the result of the natural course of the disease(s). Moreover, our study only included men due to the underrepresentation of women in the sub-groups created for this secondary analysis of the original randomized controlled trial (24).

Finally, we used plasma as the medium to quantify the cytokines; this component of blood samples prevents the determination of which tissue(s) act(s) as the source of decreased inflammation. Further research into the cytokines measured in this study in response to the two training modalities are warranted to identify their origin (*e.g.,* skeletal muscle, adipose tissue, splanchnic bed, immune cells) to more thoroughly understand the molecular mechanisms mediating the beneficial metabolic effects of exercise in patients with CAD with or without concurrent T2D.

## 4. Materials and Methods

*4.1. Study population*

The samples were obtained from the previously published Cardiac Rehabilitation eXercise modalities study (CRX-Modalities; Clinical trial NCT02765568), a randomized clinical trial conducted at the University of Ottawa Heart Institute (UOHI) designed to compare the efficacy of alternative cardiac rehabilitation modalities on short- and long-term physical and mental health outcomes in patients with CAD (24). This protocol was approved by the Ottawa Health Sciences Network Research Ethics Board (protocol #: 20160127-01H). The patients were randomized following the baseline phase in a 1:1:1 ratio between groups in a sex (male vs. female) and age (<60 vs. ³60 years) stratified manner using a computer-generated sequence as previously described (24). For the current study, only the participants assigned to MICT and HIIT exercise modalities from the original study were included. Participants were assigned to sub-groups with or without the co-morbidity of T2D (participants without T2D: HIIT-non-T2D and MICT-non-T2D; patients with T2D: HIIT-T2D and MICT-T2D).

The inclusion and exclusion criteria were previously described (24), with the exception that only male participants were included in the current study to avoid the confounding effects of unbalanced biological distribution since there were no female participants with T2D. Included participants were patients with CAD aged 40-74 years old who previously underwent a percutaneous coronary intervention (PCI) or CABG in the previous 4-18 weeks. These patients were also: referred to the UOHI cardiovascular rehabilitation program; able to walk autonomously; and willing to attend the on-site twice weekly cardiovascular rehabilitation

program for 12 weeks. Exclusion criteria included: current participation in structured exercise training (>2 days/week); inflammatory disease or active infection; persistent or permanent atrial fibrillation; unstable angina or established diagnosis of chronic obstructive pulmonary disease, severe mitral or aortic stenosis, or hypertrophic obstructive cardiomyopathy; unable to read French or English; or, unwilling or unable to return for follow-up visits at week 12.

*4.2. Demographic, anthropometric, functional and metabolic characteristics*

Medical information including medications was obtained from clinical databases. At baseline (pre-) and follow-up (post-; within one week of completing the 12 week intervention), the participants' height (baseline measurement only), body mass, waist circumference, body composition (bioelectrical impedance analysis; UM-041, Tanita, Roxton Industries Inc., Kitchener, Ontario), resting heart rate (HR) and blood pressure were measured, and a fasting blood sample was collected to obtain plasma and subsequently measure glucose concentration and glycated hemoglobin levels (HbA1c). A six-minute walk test (6MWT) was also performed at baseline and follow-up (24). The HIIT participants also underwent a peak graded exercise test on a treadmill to establish peak HR with an electrocardiogram (25), as is standard practice at the UOHI for higher-intensity exercise in this patient population.

*4.3. Exercise interventions*

As previously described (24), study participants were randomized to MICT or HIIT modalities. Both training protocols were 12 weeks in duration with twice weekly exercise classes performed on-site at the UOHI. Strength training programs were provided to the participants regardless of group assignment, and they were encouraged to perform one weekly session of

strength training exercises on their own (*e.g.,* shoulder press and raise, bent over row, elbow flexion and extension, chest press, squat, lunge, push up, core exercises, etc.). Participants were also instructed to perform 200-400 weekly minutes of moderate-to-vigorous aerobic exercise outside their cardiovascular rehabilitation program.

4.3.1. HIIT

Classes were 45 minutes in duration, beginning with 10 minutes of warm-up at 60-70% peak HR, then four training blocks consisting of 4-minute high-intensity work periods at 85-95% peak HR interspersed with 3-minute low intensity work periods of at 60-70% peak HR for a total of 28 minutes and concluding with 5-10 minutes of cool-down at 60-70% peak HR consisting of strength and stretching exercises. The participants choose to perform the HIIT sessions either on aerobic exercise equipment (treadmill, cycle ergometer, elliptical, etc.) or aerobic dance/movement sequences. HRs were monitored directly on the exercise equipment or with a Polar HR monitor (Polar RS800CX, Polar Electro Oy, Kempele, Finland).

4.3.2. MICT

Classes were one hour in duration, beginning with 10-15 minutes of walking or low-intensity use of the exercise equipment as warm-up, then 10-15 minutes of continuous aerobic exercise (walking or jogging, cycling, elliptical or rowing) for the first 3 weeks, progressing to 30 minutes of continuous exercise for the remaining weeks at a HR 20-40 bpm above resting values, and concluding with 15 minutes of cool-down consisting of strength and stretching exercises. HRs were monitored using a Polar HR monitor, the participant's own device (e.g., Apple watch) or manual palpation.

*4.4. Cytokines quantification*

The target cytokines were measured in plasma samples collected from the participants at baseline and follow-up (week 12) using three single-plex assays (*i.e.,* CRP U-plex; SPARC and FGF21 R-plex) and two multiplex assays (*i.e.*, IL-1b and TNF-a U-plex; IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18 U-plex) from Meso Scale Discovery (Rockville, MD, USA) following the manufacturer's instructions and as previously published (26). The antibodies in all the assays were validated for target specificity with the exception of SPARC. Information about tested specificity can be found on the datasheet of the U-plex antibody products (https://www.mesoscale.com/). The intra-assay coefficient of variation for the standards were: 4.37% for IL-1b, 4.60% for TNF-a, 2.28% for CRP, 6.45% for SPARC, 2.85% for FGF21, 4.30% for IL-6, 3.10% for IL-8, 4.95% for IL-10, 6.60% for IL-13, 5.65% for IL-15 and 2.95% for IL-18. The lower and upper limits of detection of each antibody were as follows: 0.227 and 4530 pg/mL for IL-1b, 0.358 and 2900 pg/mL for TNF-a, 0.427 and 5780 pg/mL for CRP, 0.443 and 1000 ng/mL for SPARC, 0.58 and 20 000 pg/mL for FGF21, 0.1 and 2060 pg/mL for IL-6, 0.06 and 2010 for IL-8, 0.08 and 3610 pg/mL for IL-10, 2.26 and 2440 for IL-13, 0.6 and 3000 pg/mL for IL-15, and 0.28 and 39 100 pg/mL for IL-18.

*4.5. Statistical analyses*

The age of the participants in the four different groups were compared using two-way ANOVA, with the factors being type of training and T2D status, and Šidák's multiple comparison was used as a post-hoc test. Data relating to the characteristics of the participants (BMI, waist circumference, body fat %, systolic and diastolic blood pressure, resting heart rate (RHR),

fasted blood glucose, glycated hemoglobin (HbA1c), as well as 6MWT results were first assessed for normality and lognormality in the sub-groups using the D'Agostino-Pearson omnibus K2 test. When normality was not achieved, but the data followed a lognormal distribution, they were transformed to their logarithms before further analyses. The data were analyzed by three-way ANOVA with repeated measures or mixed effects analysis (when time-points were missing) with the different factors being training type, T2D status and time-point of intervention (baseline and follow-up). Šidák's multiple comparisons was used as a post-hoc test.

Plasma cytokine levels were first analyzed using the 'identify outliers' function of Prism 9 with the ROUT method (Q=1%) by sub-group. Any identified outlier was then omitted during subsequent analyses. The remaining data were then assessed for normality and lognormality using the D'Agostino-Pearson omnibus K2 test as previously described and the necessary transformations were performed. The cytokine concentrations or their logarithm were then analyzed using 3-way ANOVA with repeated measures or mixed effects analysis when data points for pre- or post-training intervention were missing. Šidák's multiple comparisons was used as a post-hoc test. When applicable, the transformed variables were used for statistical analyses; however, non-adjusted values are reported in the results for descriptive purposes. All statistical analyses were performed with Prism 9 software from GraphPad (San Diego, CA, USA). A p-value $<0.05$ was considered significant for all tests.

## 5. Conclusions

Although our conclusions are limited due to low statistical power in some groups and high inter-individual variability, these data regarding plasma concentrations of cytokines in patients with CAD and T2D remains of high value for comparison and/or compilation in future studies/meta-analyses. While we did not find an increased effect of the HIIT-based exercise intervention program on reducing the cytokines in the circulation of the study participants compared to the MICT-based intervention, both training types reduced inflammation in these patients. This effect was preserved in patients with both CAD and T2D, and even more pronounced for FGF21 and IL-6, suggesting that T2D does not interfere with the positive effects of exercise on inflammatory markers. Our findings highlight the potential for both HIIT and MICT to help patients with CAD and co-morbidities such as T2D to reduce their risk factor of adverse cardiac events by regulating the levels of certain inflammation-related markers such as the cytokines discussed (*i.e.,* FGF21, IL-6, IL-8, IL-10 and Il-18).

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*Table 1 .Characteristics of the study participants pre- and post-training intervention.*

| | **Non-T2D groups** | | | | **T2D groups** | | | | **p-values** | | | **p-values interactions** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HIIT (n=14) | | MICT (n=13) | | HIIT (n=5) | | MICT (n=6) | | Effect of time | Effect of training modality | Effect of T2D | Time and training modality | Time and T2D | Training modality and T2D | Time, training modality and T2D |
| | Pre- | Post- | Pre- | Post- | Pre- | Post- | Pre- | Post- | | | | | | | |
| **Age (years)** | 60.2±7.8 | | 62±7.2 | | 61.8±3.6 | | 56.8±7.5 | | - | 0.4917 | 0.5410 | - | - | 0.1987 | - |
| **BMI (kg/m$^2$)** | 30.5±5.9 | 30.2±6.4 | 29.9±3.4 | 29.7±3.4 | 30.9±2.9 | 31.4±2.7 | 32.2±10.2 | 30.6±9.8 | 0.2400 | 0.6903 | 0.6856 | 0.1113 | 0.2394 | 0.8078 | 0.1903 |
| **Waist circumference (cm)** | 104.1±11.9 | 104.8±13.7 | 104.3±8.3 | 111.1±23.0 | 107.7±7.6 | 109.2±7.5 | 107.9±22.5 | 105.4±20.9 | 0.6926 | 0.7188 | 0.5141 | 0.0516 | 0.7489 | 0.7136 | 0.2461 |
| **Body fat (%)** | 28.6±6.7 | 27.7±5.5 | 28.4±5.5 | 28.8±4.7 | 30.6±4.7 | 29.8±4.1 | 28.0±5.9 | 25.2±4.2 | 0.1101 | 0.3776 | 0.9267 | 0.6315 | 0.3106 | 0.3234 | 0.3348 |
| **Systolic blood pressure (mmHg)** | 120.3±13.5 | 123.5±14.6 | 127.2±14.2 | 125.1±12.1 | 116.2±9.8 | 125.2±20.5 | 121.5±15.3 | 115.3±16.3 | 0.7484 | 0.9965 | 0.3800 | 0.0455 | 0.7545 | 0.5190 | 0.3781 |
| **Diastolic blood pressure (mmHg)** | 79.9±9.0 | 83.8±10.3 | 81.3±11.1 | 81.9±7.5 | 76.6±6.0 | 80.2±8.7 | 76.5±9.4 | 71.8±10.5 | 0.6853 | 0.3847 | 0.0904 | 0.1374 | 0.8070 | 0.4427 | 0.6484 |
| **Resting heart rate (beats/min)** | 58.9±8.3 | 58.1±7.1 | 62.8±13.6 | 57.7±10.0 | 64.6±8.3 | 58.6±8.6 | 65.1±10.0 | 58.5±9.7 | 0.0129 | 0.8863 | 0.5471 | 0.5946 | 0.2847 | 0.7671 | 0.4291 |
| **Fasting blood glucose (mmol/L)** | 5.23±0.46 | 5.41±0.51 | 5.55±0.44 | 5.20±0.60 | 5.86±1.39 | 5.90±1.39 | 6.37±2.93 | 7.03±0.47 | 0.4146 | 0.1421 | 0.0015 | 0.9887 | 0.2036 | 0.2055 | 0.1580 |
| **HbA1c (%)** | 5.75±0.25 | 5.87±0.31 | 5.53±0.45 | 5.72±0.34 | 6.72±1.54 | 6.90±1.00 | 6.85±1.29 | 6.57±1.09 | 0.9363 | 0.6466 | <0.0001 | 0.7247 | 0.2255 | 0.6669 | 0.4785 |
| **6-minute walking test (m)** | 577.8±52.2 | 620.1±58.4 | 596.5±64.4 | 639.1±73.3 | 533.7±84.3 | 571.5±73.4 | 549.4±58.4 | 617.3±98.4 | <0.0001 | 0.2818 | 0.0842 | 0.2334 | 0.1098 | 0.1434 | 0.2246 |

High intensity interval training (HIIT), moderate intensity continuous training (MICT), non-T2D groups refers to patients with CAD without T2D and T2D groups refer to patients with CAD and this co-morbidity, body mass index (BMI), glycated hemoglobin (HbA1c), at baseline (pre-) and follow-up (post-) of a 12-week training intervention. Data are shown ± SD.

## Figures

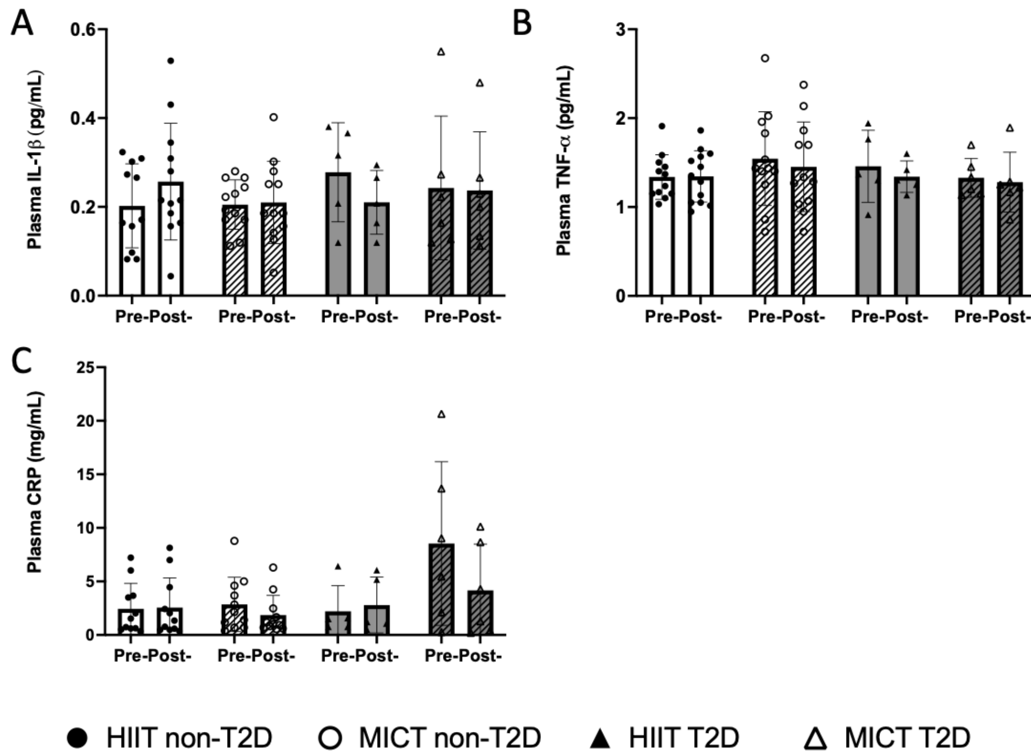


*Figure 1. Circulating cytokine concentrations in participants with CAD with or without T2D in response to a HIIT- or MICT-based rehabilitation program. Plasma cytokine concentrations at rest in men with CAD without (non-T2D) or with the T2D co-morbidity (T2D) before (pre) and after (post) a supervised 12-week training intervention consisting of either HIIT or MICT. (A) IL-1b, (B) TNF-a, and (C) CRP. HIIT non-T2D group (black circle): n=23 for IL-1b, n=25 for TNF-a, n=22 for CRP, MICT non-T2D group (white circle): n=24 for IL-1b and TNF-a, n=22 for CRP, HIIT T2D group (black triangle): n=10 for all, MICT T2D group (white triangle): n=12 for all. Data are shown individually and bars represent the average ± SEM.*

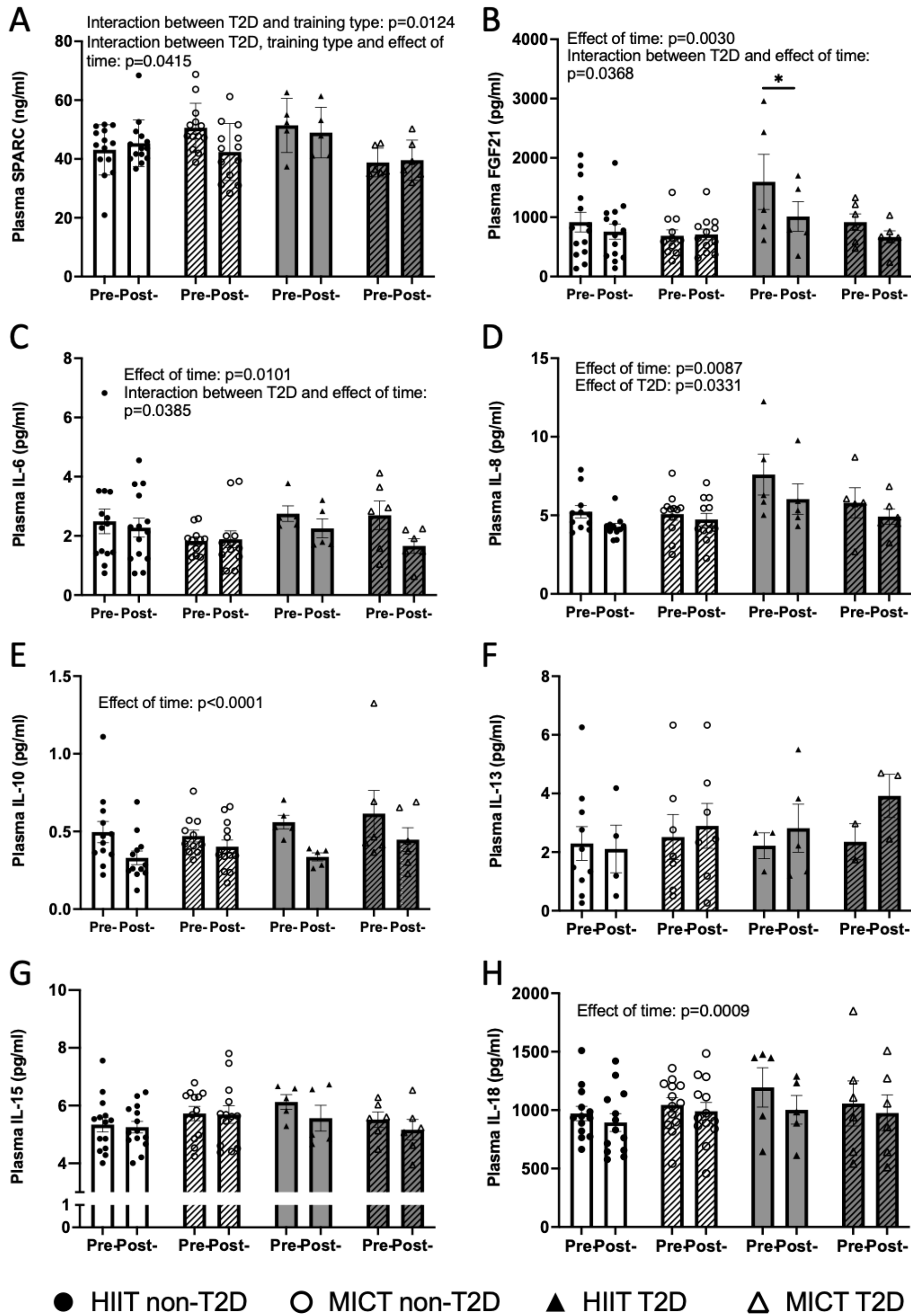


*Figure 2. Circulating cytokine concentrations in participants with CAD with or without T2D in response to a HIIT- or MICT-based rehabilitation program. Plasma cytokine concentrations at rest in men with CAD without (non-T2D) or with the T2D co-morbidity (T2D) before (pre) and after (post) a supervised 12-week training intervention consisting of either HIIT or MICT. (A) SPARC, (B) FGF21, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) IL-15, and (H) IL-18. HIIT non-T2D group (black circle): n=28 for SPARC, FGF21, IL-6 and IL-15, n=26 for IL-18, n=24 for IL-*

*10, n=22 for IL-8, n=14 for IL-13. MICT non-T2D group (white circle): n=26 for SPARC and IL-18, n=25 for IL-15, n=24 for IL-8 and IL-10, n=23 for FGF21 and IL-6, n=14 for IL-13. HIIT T2D group (black triangle): n=10 for SPARC, FGF21, IL-6, IL-8, IL-10, IL-15 and IL-18, n=8 for IL-13. MICT T2D group (white triangle): n=12 for SPARC, FGF21, IL-6, IL-10, IL-15 and IL-18, n=11 for IL-8, n=5 for IL-13. Effect of time refers to differences pre- and post-intervention regardless of training type, type of training effect refers to variations between HIIT and MICT regardless of diabetes status, effect of T2D refers to differences between groups of patients with CAD only or with the co-morbidity of T2D. *$p<0.05$ post- in comparison to pre-training intervention. Data are shown individually and bars represent the average ± SEM.*

## Appendix

*Appendix 1. List of medications taken by the participants at baseline (pre-) and follow-up visits (post-intervention).*

| | **Non-T2D groups *n* (%)** | | | | **T2D groups *n* (%)** | | | |
|---|---|---|---|---|---|---|---|---|
| | HIIT (n=14) | | MICT (n=13) | | HIIT (n=5) | | MICT (n=6) | |
| | Pre- | Post- | Pre- | Post- | Pre- | Post- | Pre- | Post- |
| **Ace-inhibitor** | 7 (50) | 7 (50) | 5 (38) | 5 (38) | 2 (40) | 1 (20) | 4 (67) | 4 (67) |
| **Acetylsalicylic acid** | 13 (93) | 13 (93) | 10 (77) | 10 (77) | 3 (60) | 3 (60) | 5 (83) | 5 (83) |
| **Calcium-antagonist** | 2 (14) | 2 (14) | 1 (8) | 1 (8) | 2 (40) | 2 (40) | 0 (0) | 0 (0) |
| **Statins** | 12 (86) | 12 (86) | 11 (85) | 11 (85) | 5 (100) | 5 (100) | 6 (100) | 6 (100) |
| **Diuretics** | 1 (7) | 1 (7) | 1 (8) | 1 (8) | 0 (0) | 1 (20) | 0 (0) | 0 (0) |
| **Clopidogrel** | 0 (0) | 1 (7) | 1 (8) | 1 (8) | 0 (0) | 0 (0) | 2 (33) | 2 (33) |
| **Nicotine replacement** | 0 (0) | 0 (0) | 1 (8) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| **Anti-platelet** | 11 (79) | 10 (71) | 8 (62) | 8 (62) | 3 (60) | 3 (60) | 3 (50) | 3 (50) |
| **β-blocker** | 12 (86) | 11 (79) | 8 (62) | 9 (69) | 5 (100) | 5 (100) | 5 (83) | 5 (83) |
| **Nitrate** | 6 (43) | 6 (43) | 8 (62) | 8 (62) | 2 (40) | 2 (40) | 3 (50) | 3 (50) |
| **Angiotensin receptor blocker** | 2 (14) | 2 (14) | 1 (8) | 1 (8) | 1 (20) | 1 (20) | 1 (17) | 1 (17) |
| **Anti-depressant** | 1 (7) | 1 (7) | 2 (15) | 2 (15) | 0 (0) | 0 (0) | 1 (17) | 1 (17) |
| **Insulin** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (40) | 3 (60) | 1 (17) | 2 (33) |
| **Oral hypoglycemiant** | 0 (0) | 0 (0) | 1 (8) | 1 (8) | 3 (60) | 3 (60) | 2 (33) | 2 (33) |
| **Fibrate** | 0 (0) | 0 (0) | 0 (0) | 1 (8) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| **Coumadin** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (20) | 0 (0) | 0 (0) |

High intensity interval training (HIIT), moderate intensity continuous training (MICT), non-T2D groups refers to patients with CAD without T2D and T2D groups refer to patients with CAD and this co-morbidity, at baseline (pre-) and follow-up (post-) of a 12-week training intervention.

# Chapter 4

# Myokine Secretion Following an Aerobic Exercise Intervention in Individuals with Type 2 Diabetes With or Without Exercise Resistance

The aim of this study was to determine whether an aerobic exercise intervention would modulate myokine release in circulation, as well as muscle secretion as a function of T2D status, and the ability in patients with T2D to show improvements in glucose homeostasis with exercise. To attain these objectives, we used samples that were shared with us from the Resist study performed by Dr. Sparks' team at the Advent Health Translational Research Institute on Metabolism and Diabetes in Orlando (NCT01911104). Their team shared serum samples and biopsy-derived primary muscle cells collected before and after a 10-week aerobic exercise intervention for us to explore how chronic exercise affected myokine secretion in the circulation and locally in the muscle cells of the participant population. We assessed the effect of the intervention on secretion patterns of SPARC, FGF21, BDNF, IL-6, IL-8, IL-10, IL-13, IL-15 and IL-18 in the serum, the cell lysates at protein and mRNA levels, as well as after treatment with *an intro* mimetic of muscle contraction. Our research allowed for comparative analysis between metabolic improvements or lack thereof with increased physical activity in patients with T2D and changes in myokine secretion in comparison to individuals without T2D.

This study was published in the International Journal of Molecular Sciences as a part of the Special Issue Molecular Insights into the Role of Exercise in Disease and Health.

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*Article*

# Myokine Secretion Following an Aerobic Exercise Intervention in Individuals with Type 2 Diabetes With or Without Exercise Resistance

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**Abstract:** Type 2 diabetes (T2D) is characterized by muscle metabolic dysfunction that exercise can minimize, but some patients do not respond to an exercise intervention. Myokine secretion is intrinsically altered in patients with T2D, but the role of myokines in exercise resistance in this patient population has never been studied. We sought to determine if changes in myokine secretion were linked to the response to an exercise intervention in patients with T2D. Participants followed a 10-week aerobic exercise training intervention and patients with T2D were grouped based on muscle mitochondrial function improvement (responders versus non-responders). We measured myokines in serum and cell culture medium of myotubes derived from participants pre- and post-intervention, and in response to an *in vitro* model of muscle contraction. We also quantified the expression of genes related to inflammation in the myotubes pre- and post-intervention. No significant differences were detected depending on T2D status or response to exercise in the biological markers measured, with the exception of modest differences in expression patterns for certain myokines (IL-1β, IL-8, IL-10 and IL-15). Further investigation into the molecular mechanisms involving myokines may explain exercise resistance with T2D, however the role in metabolic adaptations to exercise in T2D requires further investigation.

**Keywords:** myokines; type 2 diabetes; obesity; aerobic exercise; training intervention; skeletal muscle; exercise resistance; inflammation.

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## 1. Introduction

Type 2 diabetes (T2D) is a chronic, non-communicable disease associated with poor nutritional habits, reduced physical activity and increased sedentary behaviour[1-3]. In patients with T2D, some of the earliest metabolic manifestations of the disease are the development of skeletal muscle insulin resistance and mitochondrial dysfunction[4, 5]. Exercise interventions are a popular addition and alternative to traditional pharmacological treatments for the management of T2D, as they have been shown to increase skeletal muscle mitochondrial oxidative capacity and improve insulin sensitivity[6, 7]. The AMP activated protein kinase (AMPK) pathway is central to muscle signaling during exercise, since it serves as an energy sensor responding to the rise in AMP/ATP ratio that occurs during muscle contraction[8]. Muscle contraction causes AMPK phosphorylation and consequent downstream activation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), an important

modulator of mitochondrial dynamics that enhances mitochondrial oxidative capacity (OXPHOS)[9]. PGC1α can also be activated by sirtuins in response to exercise, causing a remodeling of the electron transport chain complexes to enhance OXPHOS efficiency[10, 11]. In addition to these intracellular signaling events, the metabolism of skeletal muscle cells is regulated by extracellular signaling mechanisms during exercise.

Indeed, skeletal muscle is an important secretory organ, releasing peptides called myokines in the muscle interstitium during muscle contraction[12]. Myokines can be regulated both at the transcript and protein levels by acute and chronic exercise and they have been shown to greatly impact the regulation of insulin sensitivity and mitochondrial function in skeletal muscle[13]. For example, interleukin (IL)-6, IL-10, IL-13, IL-15, IL-18, brain derived neurotrophic factor (BDNF), fibroblast growth factor 21 (FGF21), and secreted protein acidic and rich in cysteine (SPARC) act locally in muscle cells by improving mitochondrial function, insulin-independent glucose uptake and/or insulin sensitivity[14-28]. Many of these myokines modify skeletal muscle metabolism through the AMPK pathway following exercise[20, 24, 28, 29]. As such, exposing L6 rat skeletal muscle cells to IL-15 at physiological concentrations enhances mitochondrial function in parallel to the activation of the AMPK pathway and increased glucose uptake[30]. Some myokines are thought to play an endocrine role in regulating metabolic adaptations to exercise. IL-6 has been shown to influence insulin secretion[31-34], and IL-13 can positively modulate insulin secretion by improving β-cells function and/or limiting cytokine-induced β-cell apoptosis[35]. Conversely, FGF21 treatment improved hepatic and whole-body insulin sensitivity in a mouse model of diet-induced obesity[36]. FGF21 has also been shown to regulate lipolysis in adipose tissue[37]. IL-15 treatment in adipocytes results in increased secretion of adiponectin[38], an adipokine known to promote insulin sensitivity, as well as increased mobilisation and catabolism of circulating lipids[39]. Therefore, myokines are important modulators of both skeletal muscle and whole-body metabolism in response to an exercise intervention.

Some subjects with T2D experience no improvements in glucose homeostasis in response to exercise interventions, a phenomenon termed 'exercise resistance'. For example, after an exercise intervention protocol of 12-16 weeks of aerobic moderate-intensity continuous training in a cohort of around one hundred subjects with pre-diabetes or T2D, roughly one third of participants showed no improvements in glucose control[40]. Similar results were found in women with impaired glucose tolerance after a 12-week high-intensity interval training or resistance training intervention[41]. This supports the notion of non-responders to glucose control improvements with different types of interventions. Moreover, *in vivo* and *ex vivo* assessment of mitochondrial function via the measurement of phospho-creatine (PCr) recovery rate in patients with T2D following a 10-week exercise intervention established that the lack of improvement in insulin sensitivity correlates with the absence of changes in mitochondrial function[42]. The primary outcome of the RESIST study (NCT01911104) showed that participants who qualified as non-responders had higher expression patterns of genes linked to antioxidants, mitochondrial metabolism and insulin signaling at baseline compared to responders[43]. These epigenetic molecular regulatory mechanisms were maintained in biopsy-derived primary muscle cells cultured *in vitro*, suggesting a cell-autonomous process.

Many other factors such as duration of T2D, age, genetics and epigenetics contribute to the exercise response in patients with T2D, regardless of exogenous factors such as the type and duration of the exercise intervention[44]. Although myokine secretion is intrinsically altered in patients with T2D[45], the myokine profiles of responders and non-responders to the beneficial effects of exercise has not yet been compared after a training intervention. Altered myokine secretion in response to acute and chronic exercise could therefore contribute to the impaired improvements in glucose homeostasis and mitochondrial function in patients with T2D non-responsive to an exercise intervention.

As myokines are mainly cytokines that can be secreted by other tissues, measurement of their levels in plasma or serum does not adequately represent the secretion of myokines by skeletal muscle. That said, when isolated from muscle biopsies of patients with T2D and cultured *in vitro*, human primary skeletal muscle cells maintain their insulin resistance, mitochondrial dysfunction, and altered myokine secretion[45-49]. Isolated muscle cells can be differentiated into myotubes that resemble mature muscle cells. This model using samples obtained directly from clinical participants is ideal for the study of myokines secreted specifically by skeletal muscle cells during T2D without the interference of surrounding tissues[50]. In addition, myotubes can be subjected to electrical pulse stimulations (EPS), which mimic muscle contraction *in vitro*[51]. EPS has been shown to induce metabolic adaptations similar to those observed in muscle after exercise, while also increasing myokine secretion[52, 53]. Most studies using EPS with human primary muscle cells employed long durations of stimulations (4-48h) at low frequencies (≤2Hz) resulting in improved oxidative functions, mitochondrial biogenesis, glucose uptake, insulin sensitivity and increased release of myokines in the cell culture supernatant[52-57]. That being said, these stimulation conditions do not translate to muscle contraction *in vivo*, as the stimulus is considerably longer than an average bout of exercise would last. To mimic acute muscle contraction, Li *et al.* treated C2C12 mouse muscle cells with 1h of EPS at 20V, 1Hz frequency with 24ms impulses every 976ms (24ms on the second), resulting in increased cell surface GLUT4 localization through AMPK, and downstream TBC1D1 and AS160 (TBC1D4) signaling[58]. C2C12 myotubes have a greater EPS contractile activity than human primary muscle cells because of their more rounded morphology[59]. This characteristic of human myotubes warrants validation of these stimulation conditions prior to any inference between *in vivo* exercise and *in vitro* myotube contraction in clinically-derived cell lines.

This study is a secondary analysis of the randomized controlled trial NCT01911104, which focused on the epigenetic mechanisms occurring in skeletal muscle and contributing to exercise resistance in patients with T2D. We sought to determine if myokine signaling adaptations to exercise and muscle contraction differed according to T2D status, and/or as a function of the ability to respond to an exercise intervention in the case of participants living with T2D as assessed by their rate of muscle PCr recovery. To this end, we measured myokines in serum samples of participants pre- and post-intervention, as well as in cell culture supernatant of biopsy-derived human primary muscle cells collected pre- or post-intervention, and stimulated or not with an *in vitro* model of muscle contraction (EPS). We also measured the expression of myokines in the isolated muscle cells pre- and post-intervention. We hypothesized that local myokines secretion (measurements in cell culture supernatant samples) might be dysregulated in the group of patients with T2D that do not manifest metabolic improvements with exercise training in comparison to those who benefit from the intervention.

## 2. Results

### *2.1. Patient Characteristics*

Anthropometric and metabolic characteristics of the participants in this sub-study can be found in Table 1; full cohort characteristics have been previously published[42]. No differences were found between groups for age, BMI, fat mass, lean mass, $VO_{2max}$, and adherence to the program (No T2D 79.0%; T2D responders 76.3%; T2D non-responders 76.8%). The apparent higher mean age in the T2D responders group and mean BMI in the No T2D group are due to a single high value in each group and the p-values were not significant (p=0.1016 and p=0.5109, respectively). There were no significant differences amongst the groups pre- compared to post-intervention for BMI, fat mass, and $VO_{2max}$. Glycated hemoglobin (HbA1c) levels were higher in the two groups of participants with T2D compared to the no T2D group

independently of the intervention status (p=0.0007). The effect of the intervention was significant on HbA1c values (p=0.0342), and a significant interaction was detected between groups and the effect of the intervention (p=0.0131). In the group of patients with T2D classified as non-responders to the exercise intervention, HbA1c levels were significantly higher post-intervention compared to pre-intervention (p=0.0012). This effect on HbA1c levels is associated with the interruption of glucose lowering treatment in participants with T2D. An effect of the intervention was found for changes in lean mass across all groups (p=0.0013) and the increase reached significance in the T2D non-responders group in the post-hoc test (p=0.0075). Although M-values during the hyperinsulinemic-euglycemic clamp did not vary significantly pre- compared to post-intervention, they were significantly different between groups (p=0.0060). Pre-intervention, M-value was lower only in the T2D responders group compared to the no T2D (p=0.0309), while post-intervention, M-value was lower in both the T2D responders (p=0.0120) and the T2D non-responders (p=0.0016) groups compared to the participants with no T2D. A significant interaction was found between the effects of group and the intervention, with the intervention showing no significant effect on PCr recovery rate in the group of participants with no T2D and T2D responders group, while a decrease occurred in the T2D non-responders group (p=0.0089). These results are in alignment with the findings from the primary outcome study, which showed a positive correlation between insulin sensitivity (M-value) and PCr recovery rate at the pre-intervention stage in the two groups of participants with T2D[42]. Both measures of energy metabolism were increased in the group of participants with T2D who qualified as non-responders in comparison to responders before the start of the intervention. These results were accompagnied with a favorable epigenetic and transcriptional profile of muscle metabolic function in the non-responders group compared to patients with T2D who qualified as responders.

Table 1. Participant characteristics pre- and post- 10-week aerobic exercise intervention.

| Group | n (m/f) | Age (years) | BMI ($kg/m^2$) | | HbA1c (%) | | Fat mass (kg) | | Lean mass (kg) | | M-value (mg/kg/min) | | $VO_{2max}$ (ml/kg/min) | | PCr recovery rate (1/s) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pre | post | pre | post | pre | post | pre | post | pre | post | pre | post | pre | post |
| No T2D | 7 (2/5) | 45.3 ± 6.3 | 37.2 ± 6.7 | 37.4 ± 5.9 | 5.5 ± 0.5 | 5.7 ± 0.4 | 44.1 ± 12.0 | 44.5 ± 11.3 | 51.2 ± 12.8 | 51.9 ± 12.2 | 168.0 ± 95.3 | 231.4 ± 116.5 | 22.1 ± 6.1 | 23.6 ± 5.4 | 0.025 ± 0.014 | 0.022 ± 0.008 |
| T2D responders | 9 (6/3) | 53.4 ± 7.1 | 34.8 ± 3.6 | 34.6 ± 3.9 | 7.6 ± 0.8††† | 7.9 ± 0.9††† | 40.2 ± 8.9 | 39.7 ± 9.4 | 58.7 ± 10.0 | 59.1 ± 9.7 | 59.8 ± 51.5† | 78.6 ± 43.1†† | 23.2 ± 4.1 | 25.1 ± 5.1 | 0.015 ± 0.003‡‡ | 0.020 ± 0.004 |
| T2D non-responders | 6 (3/3) | 47.2 ± 9.5 | 34.4 ± 4.5 | 34.8 ± 4.7 | 7.4 ± 0.9† | 7.9 ± 1.5**††† | 39.5 ± 9.3 | 40.6 ± 9.8 | 54.4 ± 13.9 | 56.0 ± 14.2** | 114.2 ± 54.5 | 99.7 ± 71.9†† | 24.1 ± 7.5 | 22.7 ± 4.2 | 0.026 ± 0.005 | 0.020 ± 0.004** |

Data are shown as mean ± SD. *p<0.05 and **p<0.01 in comparison to pre-intervention. †p<0.05, ††p<0.01, †††p<0.001 in comparison to no T2D group at the same time-point of intervention. ‡‡p<0.01 compared to non-responders group at the same time-point of intervention. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. BMI = body mass index. HbA1c = percentage glycated hemoglobin. $VO_{2max}$ = maximal oxygen consumption rate. PCr = phosphocreatine.

*2.2. Serum Cytokine Concentrations Following the Aerobic Exercise Intervention*

The training intervention did not affect the serum concentration of the cytokines measured in this participant population (Figure 1 A-G). There were no significant differences between groups for SPARC, FGF21, IL-10, IL-15 and IL-18 serum concentrations irrespective of the intervention status, but there was a trend towards lower circulating IL-6 levels in the T2D non-responders, although the post-hoc test was not significant (p=0.0982). Serum IL-8 concentrations were different across groups irrespective of intervention status (p=0.0074),

with a trend towards an increase in the T2D responders group in comparison to the no T2D group (p=0.0673) and a significantly higher concentration of serum IL-8 in the T2D non-responders group compared to the no T2D group (p=0.0067). IL-13 was mostly undetected in the serum samples across all groups and independently of intervention, preventing the possibility of further data analysis.

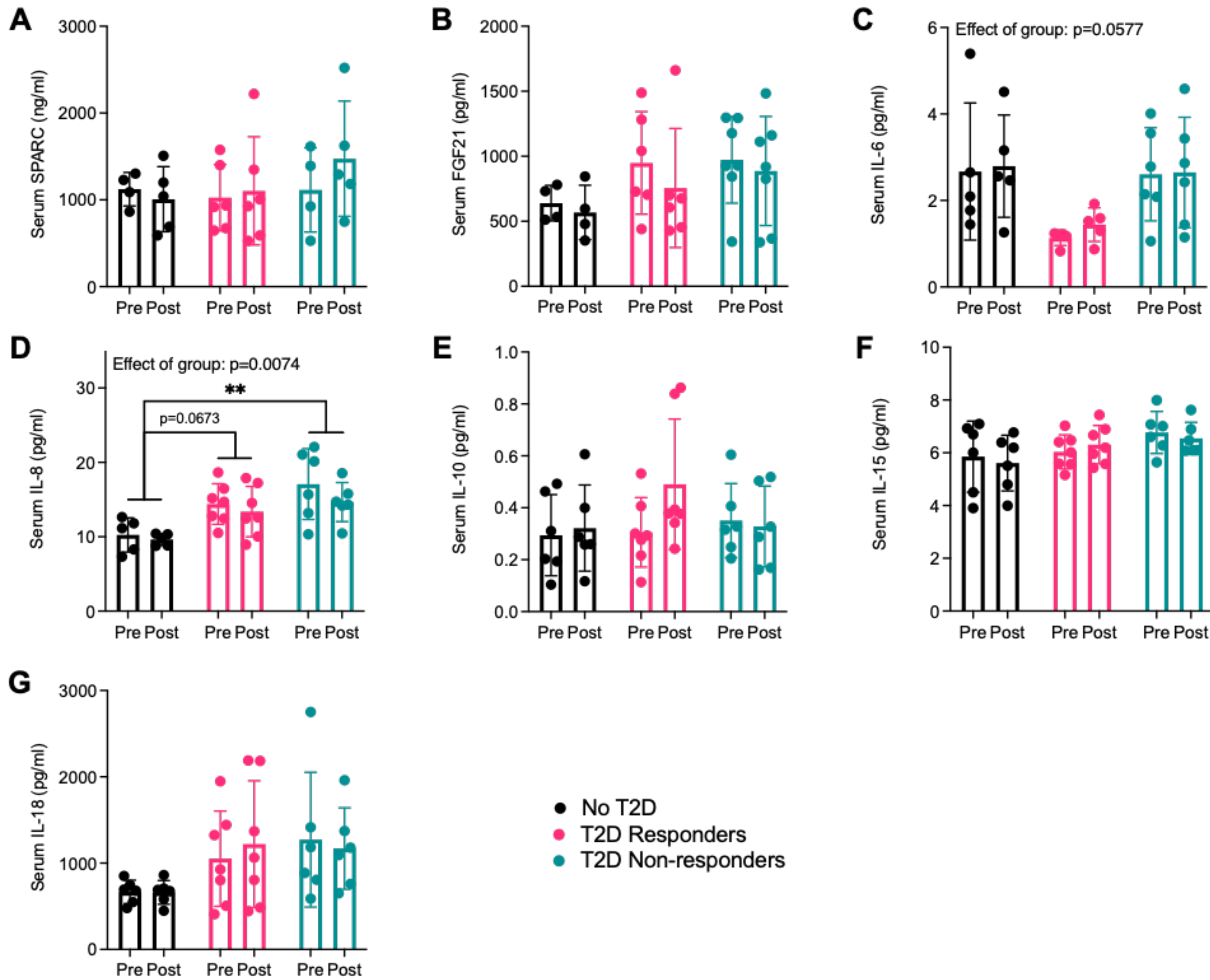


Figure 1. Variations in resting cytokine serum concentrations following the 10-week aerobic exercise intervention. (A) SPARC, (B) FGF21, (C) IL-6, (D), IL-8, (E) IL-10, (F) IL-15 and (G) IL-18 levels are presented before (pre-) and after (post) the exercise intervention. IL-13 was undetected in almost all serum samples. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. N=4-7. Data are represented as individual values and mean ± SD. **p<0.01.

*2.3. Myokines Secretion by Primary Human Muscle Cells in Response to the Intervention*

As the cytokines measured in the serum samples (Figure 1) could originate from numerous different tissues, we also measured those secreted by the primary muscle cells derived from skeletal muscle biospsies of the *vastus lateralis* collected pre- and post-intervention in a subset of the study participants to infer muscle-specific secretion of the myokines. We measured myokines in the cell culture supernatant of the biopsy-derived muscle cells exposed to the myotubes for 1h, and found no differences between pre- and post-intervention myokine concentrations (Figure 2 A-F). Most of the candidate myokines measured were detected in the

cell culture supernatant (SPARC, IL-6, IL-8, IL-10, IL-15 and IL-18), but FGF21, BDNF, and IL-13 were mostly undetected independently of group or intervention status of the participants from which the cells originated.

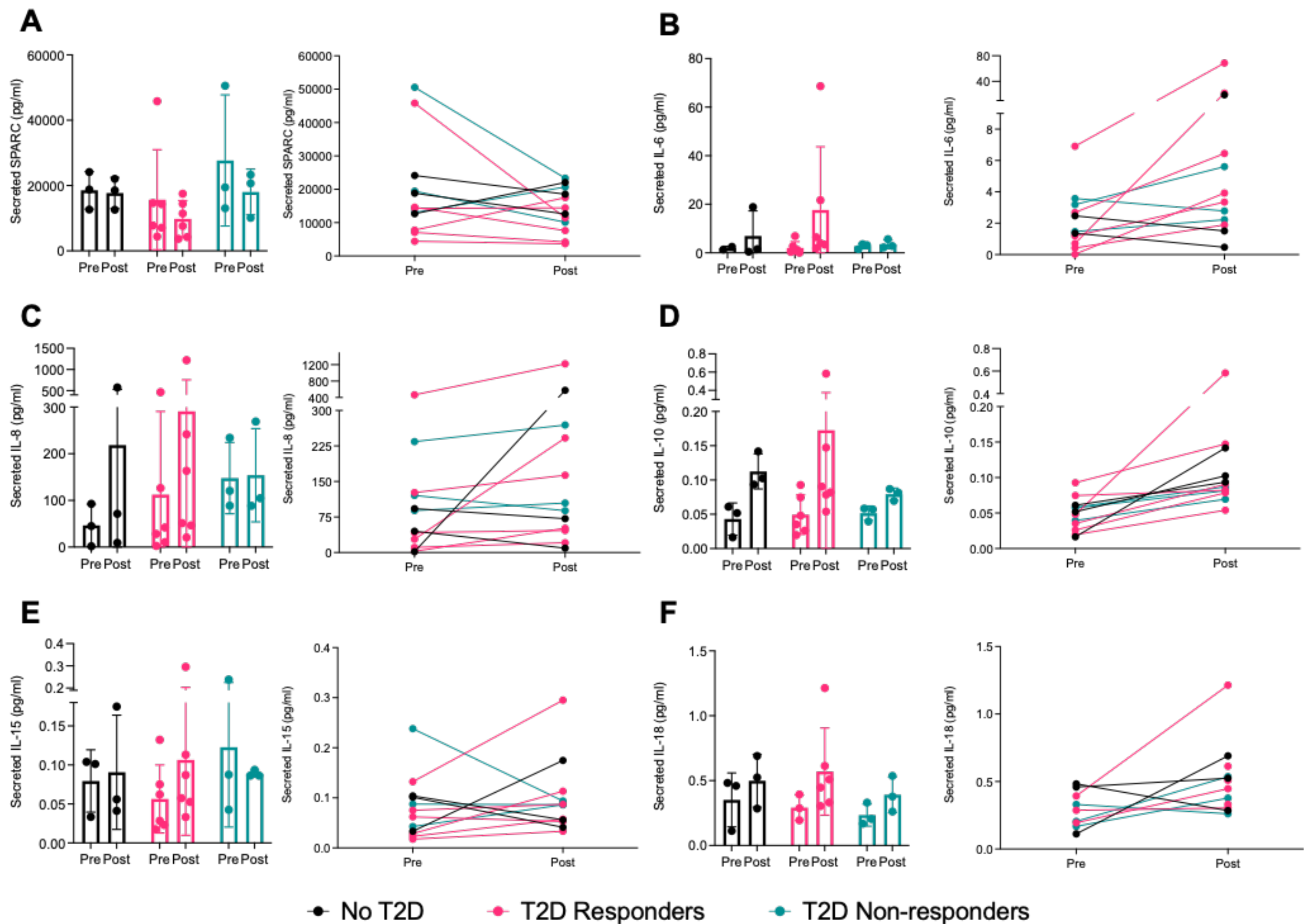


Figure 2. Variations in myokine secretion in cell culture supernatant of human primary myotubes in response to the 10-week aerobic exercise intervention. Myokines were measured by R-plex and U-plex assays (MSD, Maryland, USA). (A) SPARC, (B) IL-6, (C) IL-8, (D) IL-10, (E) IL-15, and (F) IL-18 levels are presented before (pre-) and after (post) the exercise intervention. BDNF, FGF21, and IL-13 were mostly undetected. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. N=2-6. Data are presented as individual data points with mean ± SD and line graphs when applicable.

### *2.4 Correlation Between Myotube Secretion and Serum Myokine Concentrations*

Serum samples collected from participants for which human primary muscle cells were available were used to assess any potential correlation between myokine secretion in the circulation (peripheral) and primary muscle cells in culture (local). Data from participants across all groups pre- and post-intervention were pooled for the analyses. No significant correlations were found between serum and cell culture supernatant concentrations for any of the cytokines/myokines assessed (SPARC, IL-6, IL-8, IL-10, IL-15, and IL-18; Figure 3 A-F).

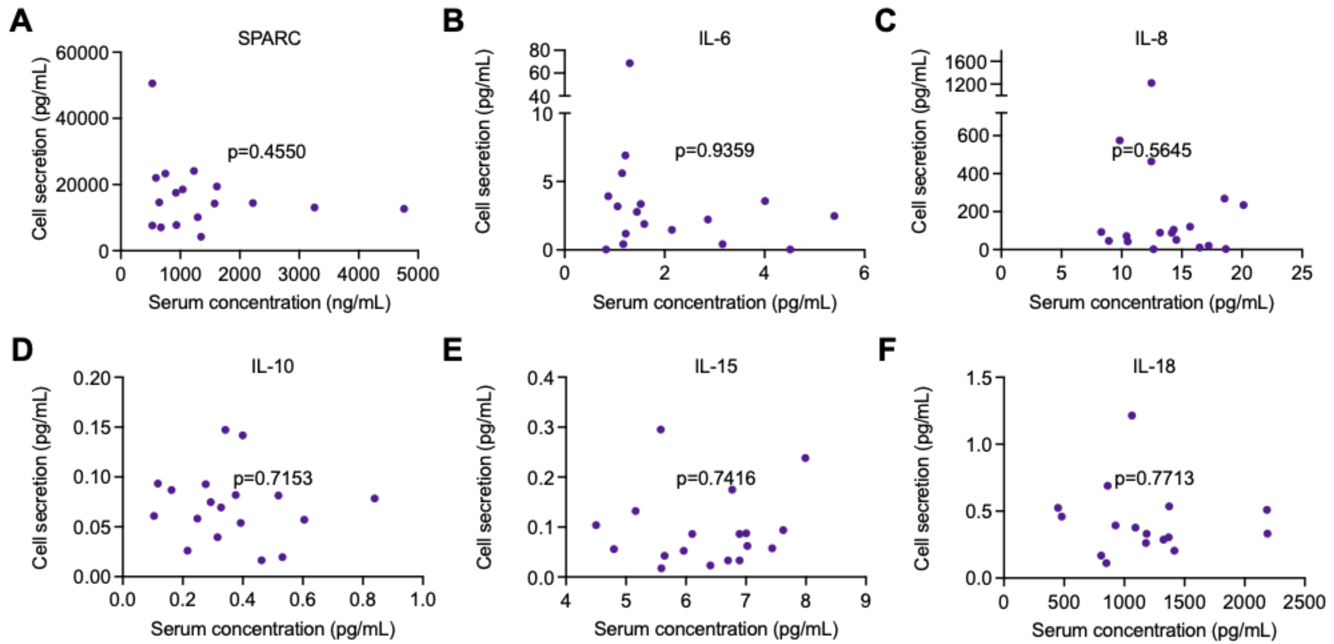


Figure 3. Correlation analysis between myokine secretion in cell culture supernatant of human primary myotubes and serum concentrations of cytokines from the participants. Myokines were measured by R-plex and U-plex assays (MSD, Maryland, USA). (A) SPARC, (B) IL-6, (C) IL-8, (D) IL-10, (E) IL-15, and (F) IL-18 levels. N=16-18. Data are presented as individual data points including pre- and post-intervention values per participant.

*2.5 Effects of EPS Treatment on Primary Muscle Cell Molecular Signaling Events*

To mimic acute muscle contraction, the primary myotubes derived from muscle biopsies obtained from the participants pre- and post-intervention were subjected to an EPS treatment (S). Non-stimulated (NS) and electrode exposure without current (E) were used as control conditions to determine the effects of muscle cell contraction per se as opposed to the potentially pro-inflammatory effect of the carbon electrode on the myotubes[60].

2.5.1. AMPK-Pathway Activation

As mentioned previously, the AMPK signaling pathway is central to metabolic adaptations during acute muscle contraction. Upon muscle contraction and phosphorylation of AMPK, downstream phosphorylation of Acetyl-CoA Carboxylase (ACC) inhibits fatty acid synthesis, while phosphorylation of Akt Substrate 160 (AS160) contributes to glucose transporter GLUT4 translocation to the plasma membrane. In the cell lines from the no T2D group collected post-intervention, the EPS condition (S) showed significantly higher p-AMPK compared to the NS condition (Figure 4 A, p=0.0179). In the cell lines from the T2D non-responders group collected pre-intervention, the electrode treatment (E) resulted in more p-AMPK than the NS condition (Figure 4 B, p=0.0332). In the myotubes from the participants in the no T2D group pre-intervention, and in the myotubes from the T2D responders group post-intervention, the EPS condition (S) caused significantly more p-AS160 compared to the NS condition (Figure 4 A, p=0.0035 and 4 C p=0.0220, respectively). Both the EPS treatments and the study group from which the myotubes were derived (effect of group) had a significant effect on the regulation of pACC levels (Figure 4 A and D; p=0.0057 and p=0.0205, respectively). The EPS condition (S) significantly increased p-ACC in the myotubes from

participants of the no T2D group at both pre- and post-intervention (p=0.0047 and p=0.0032, respectively).

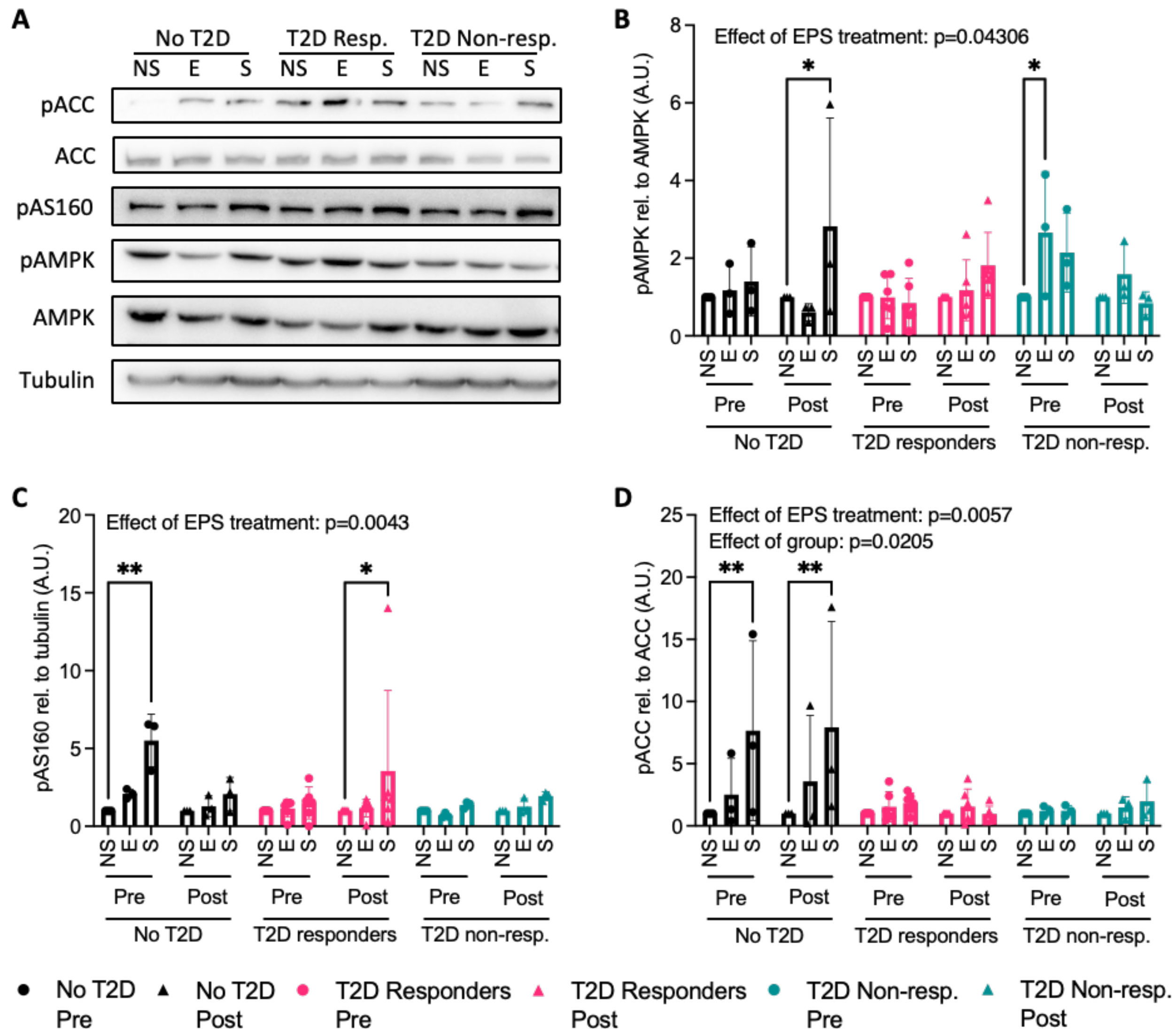


Figure 4. Effect of EPS on the phosphorylation of proteins involved in the AMPK-pathway in human primary myotubes quantified by immunoblotting. NS = non-stimulated, E = electrode exposure, S = EPS treatment, Pre = pre-intervention, Post = post-intervention. (A) Representative blot, (B) quantification of p-AMPK relative to total AMPK, (C) quantification of p-AS160 relative to tubulin, (D) quantification of p-ACC relative to total ACC. Blots were analyzed with ImageJ (NIH). No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. N=3-6. Data are shown as mean ± SD with individual data points. *p<0.05, **p<0.01.

### 2.5.2. Myokine Secretion

To determine the potential effects of EPS treatments on myokine secretion, the biopsy-derived primary cell cultures collected pre- and post-intervention were submitted to the three different conditions (NS, E, S), and myokine concentrations in the cell culture supernatant of each cell line was assessed. Myokine secretion *in vitro* was compared between EPS treatments for each cell line relative to the NS condition.

No effect of the electrode exposure (E) was observed on the secretion of the myokines quantified in the primary human muscle cells obtained from the participants in comparison

to the non-stimulated NS condition (Figure 5 A-F). There was a significant effect of groups on SPARC secretion by the myotubes (p=0.0322), but no significant differences were detected in post-hoc tests (Figure 5 A).

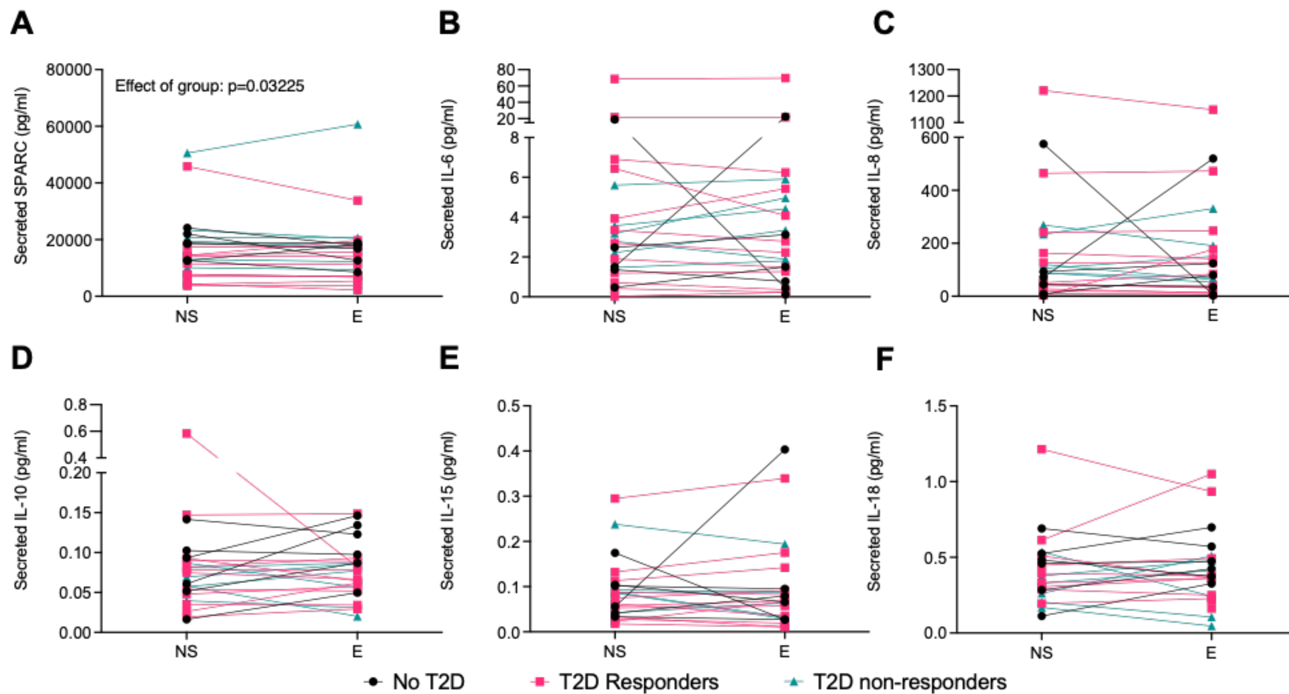


Figure 5. Effect of electrode exposure control condition of human primary myotubes collected pre- and post-intervention on myokine secretion. Myokines were measured in the cell culture supernatant by R-plex and U-plex assays (MSD, Maryland, USA). (A) SPARC, (B) IL-6, (C) IL-8, (D) IL-10, (E) IL-15, and (F) IL-18. BDNF, FGF21, and IL-13 were mostly undetected. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. Non-stimulated = NS, exposed to the electrode apparatus for 1h = E. N=6-12. Data are shown individually with lines connecting the NS and E conditions for one cell line.

Treatment of the myotubes derived from the participants in all groups with the 1h EPS protocol (stimulated; S) did not significantly alter myokine secretion in comparison to the non-stimulated (NS) condition (Figure 6 A-F). Most targeted myokines were detected in the cell culture supernatant (SPARC, IL-6, IL-8, IL-10, IL-15 and IL-18), but some were mostly undetected and their secretion pattern could not be analyzed (BDNF, FGF21 and IL-13).

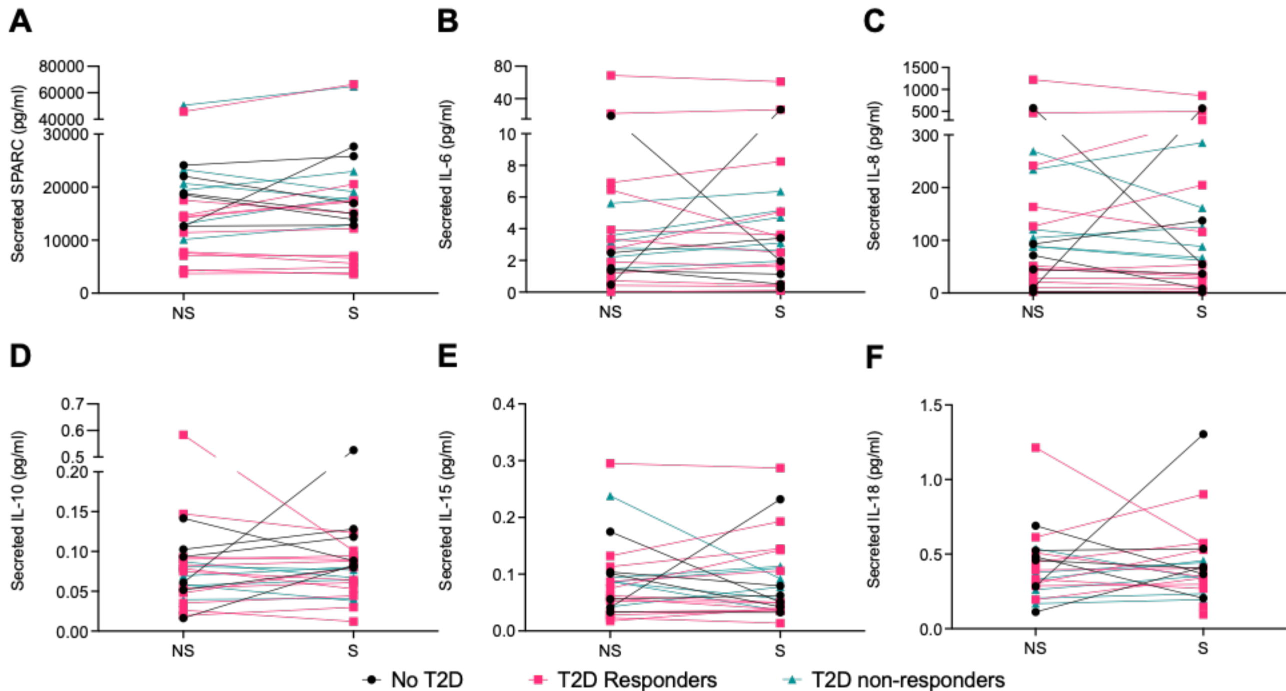


Figure 6. Effect of EPS treatment of human primary myotubes collected pre- and post-intervention on myokine secretion. Myokines were measured in the cell culture supernatant by R-plex and U-plex assays (MSD, Maryland, USA). (A) SPARC, (B) IL-6, (C) IL-8, (D) IL-10, (E) IL-15, and (F) IL-18. BDNF, FGF21, and IL-13 were mostly undetected. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. N=6-12 Data are shown individually with lines connecting the non-stimulated (NS) and EPS stimulated (S) conditions for one cell line.

### *2.6 Regulation of Gene Expression in Human Primary Myotubes Following the Intervention*

Gene expression analyses of the NS condition of myotubes from the participant groups revealed a regulatory effect of both the exercise intervention and the groups on the expression of genes related to inflammation (Figure 7 A-H). The level of mRNA expression increased between pre- and post-intervention in the T2D responders group for IL-1β (Figure 7 B, p=0.0116) and IL-8 (Figure 7 D, p=0.0114), as well as in the no T2D and T2D non-responders groups for IL-15 (Figure 7 G, p=0.0023 and p=0.0007, respectively). An interaction between the effect of the intervention and group was detected for IL-10 (p=0.0270) and IL-15 (p=0.0084) (Figure 7 E and G). Levels of IL-10 mRNA were significantly decreased post- compared to pre-intervention in the T2D non-responders group (p=0.0459), while IL-10 expression trended towards an increase post-intervention in the two other groups with no significance observed. Although IL-15 mRNA levels significantly increased in the no T2D (p=0.0023) and T2D non-responders groups (p=0.0007), there was no change in the T2D responders group.

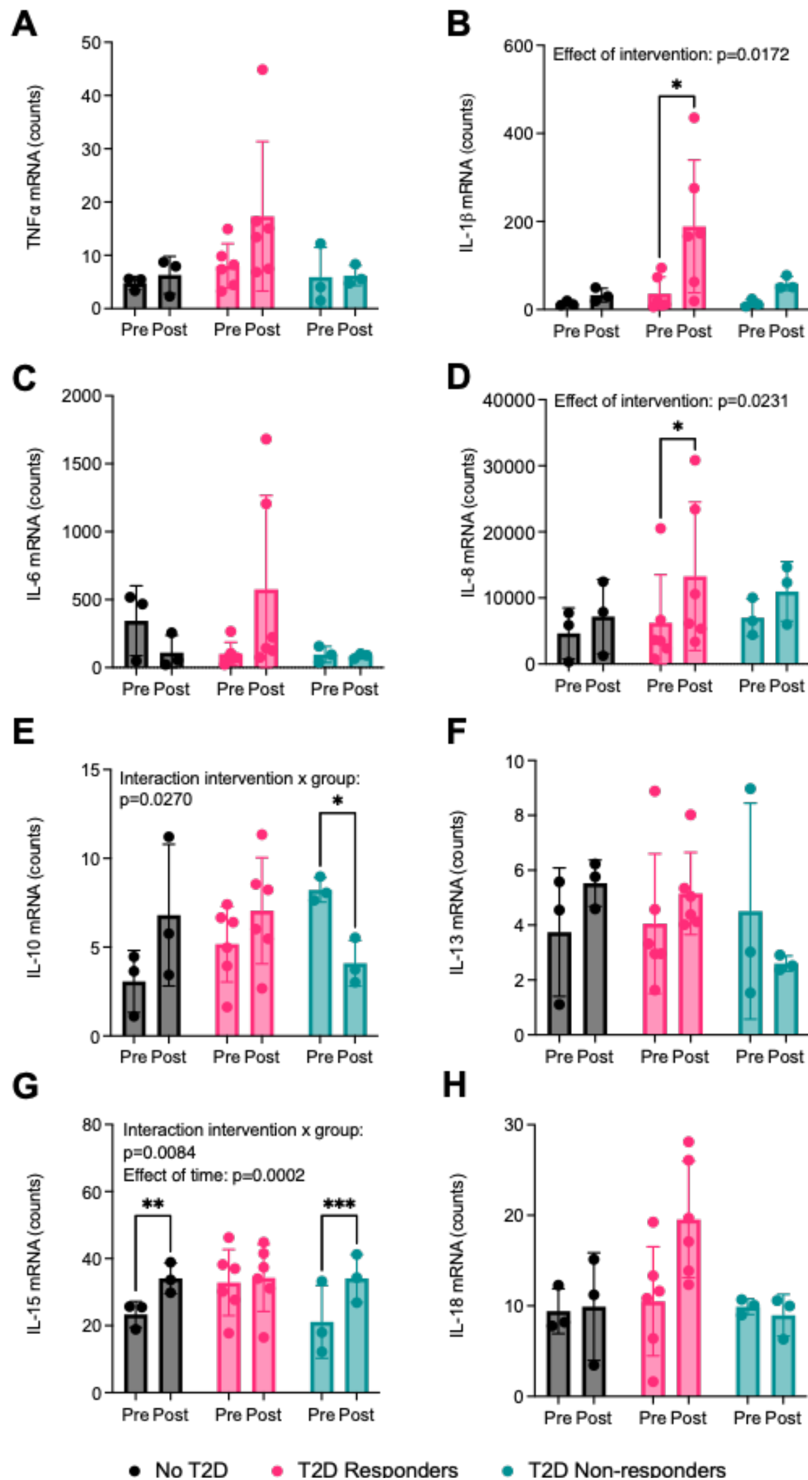


Figure 7. Effect of the 10-week aerobic exercise intervention on the mRNA expression of genes linked to inflammation in human primary myotubes as assessed by NanoString multiplex assay. Data analysis was performed with nSolver Analysis Software v4.0 and individual quantification (counts) of mRNA molecules were extracted for targets thought to be candidate myokines. Relative counts of (A) TNF-**α**, (B) IL-1**β**, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) IL-15, and (H) IL-18 quantified by multiplex assay before (pre-) and after (post-) the exercise intervention. No T2D = participants with obesity without T2D, T2D responders = patients with T2D who respond to training, T2D non-resp. = patients with T2D who do not respond to training. N=3-6. Data are shown as mean ± SD with individual data points. *p<0.05, **p<0.01, ***p<0.001.

## 3. Discussion

Aerobic exercise training is a key non-pharmacological method for the management of impaired glucose homeostasis in patients with T2D. Unfortunately, some patients living with diabetes do not benefit from the metabolic improvements associated with increased aerobic exercise. We sought to determine whether myokine signaling was a contributing factor to the mechanisms of exercise resistance in patients with T2D. We found no effect of either T2D or the capacity to respond to an exercise intervention on peripheral or local signaling adaptations at the protein level of target cytokines/myokines using serum samples and biopsy-derived primary muscle cells collected from the study participants pre- and post-exercise intervention. However, the expression pattern variations in response to the training intervention of some of the candidate myokines quantified at the mRNA level differed according to T2D and responder or non-responder status. To our knowledge, this is the first study to examine and compare local and peripheral regulation of myokine signaling in participants with obesity with or without T2D in response to an aerobic exercise intervention protocol while accounting for the ability of the patients with T2D to benefit from the intervention at the mitochondrial metabolic level. An important consideration to take into account regarding our study before discussing the results is the absence of a group of participants not undergoing the exercise training intervention. Therefore, all analyses pertaining to pre- and post-intervention states cannot be controlled for the variations in all factors assessed as a function of the natural evolution of the parameters over the 10-week time course of the observations. Moreover, the original study design did not include a group of sedentary participants with a BMI in the "healthy" range, but with characteristics similar to the groups of participants with obesity. This limitation eliminates the potential of detecting any effects of obesity on muscle signaling adaptions. Finally, another important caveat of this study is the small number of participants per group that causes a reduction in the power of the analyses performed on the data. This limitation is a consequence of the nature of the study, which serves as a secondary analysis of samples from a previously published broader clinical trial, for which we did not have access to the entire study sample pool.

We assessed the levels of circulating cytokines that are also known to be secreted by skeletal muscles in serum samples collected from the participants at rest. The only significant difference we found in the participant population was an increase in serum IL-8 levels in the two groups of patients with T2D in comparison to those without, irrespective of their response to exercise-induced metabolic adaptations. This aligns with previous characterization of circulating IL-8 levels in patients with T2D found in the literature[13, 61]. That being said, others have shown that some of the cytokines (i.e. SPARC, IL-6, IL-10, IL-13, IL-15, IL-18) we quantified in our cohort can be altered in the circulation of patients with T2D as we reviewed in 2019[13]. Since we measured circulating cytokines in serum and most of these other studies quantified cytokines in plasma, the medium assessed could explain the inconsistencies in our findings compared to others. A recent meta-analysis examining the correlation between variations in circulating levels of certain so-called exerkines found that FGF21, IL-6 and IL-10, among others, could serve as potential biomarkers to assess the effectiveness of an exercise protocol on improvements in glucose homeostasis in patients with T2D[62]. For most cytokines, variations in response to either acute or chronic exercise can be better detected in plasma samples[63], which could explain why we saw no effect of the training intervention on circulating levels of the cytokines.

The analysis of cell culture supernatant from primary muscle cells derived from the participants pre- and post-intervention revealed no differences associated with either intervention status or group. This finding contradicts those of others, who found that primary human muscle cells from patients with obesity and T2D secreted myokines differently than participants without T2D[45]. Indeed, they found higher secretion of IL-6, IL-8 and IL-15 in the muscle cells from patients with T2D in comparison to the no-T2D group. This discrepancy

could be explained by the fact that our study groups included participants with similar anthropometric values, whereas the group of patients with T2D in the Ciaraldi *et al.* study had a significantly higher BMI than their insulin sensitive counterparts. Another important factor is that the conditioned medium containing the myokines was collected after only one hour of exposure to the cells, while the other team measured the myokines after 24h and 48h of exposure. Perhaps a longer time elapsed before collection would have allowed for a better quantification of the secreted myokines. Moreover, we were unable to normalize the protein concentration of myokines in the cell culture medium to the total protein content of their respective lysates, as the samples were used for RNA isolation and the resulting protein precipitate was insoluble after processing.

To explore the potential relationship between muscle secretion of myokines and the levels of these peptides released in the circulation, we performed correlation analyses between serum and human primary muscle cell culture supernatant concentrations. There were no significant correlations between the levels of the candidate myokines in serum and their secretion by the myotubes, suggesting that the peptides quantified in the circulation of the participants are not predominantly originating from muscle tissue. As mentioned previously, serum is not the preferential medium to quantify myokine variations in response to exercise interventions as differences in their circulatory secretion is better detected in plasma samples[63]. Consequently, there is a possibility that changes in plasma concentrations could have better correlated with myotube secretion of the myokines in response to the exercise intervention. These correlation analyses further highlight the importance of measuring myokine secretion from muscle cells or tissue directly, rather than relying on the assumption that circulating levels of these peptides represent their release from muscle and not other tissues.

The acute muscle contraction *in vitro* model we employed was validated in a mouse muscle cell line (C2C12 cells), with a very different morphology in comparison to primary human muscle cells. Indeed, C2C12 muscle cells have a better contractile activity than human primary muscle cells, as they form more tubular myotubes that better resemble muscle fibers mechanically than their flat counterparts[59, 60]. Nonetheless, we saw an increase in the phosphorylation of proteins involved in signal transduction of the AMPK pathway in response to the EPS treatment (AMPK, AS160 and ACC). This molecular signaling adaptation was not paralleled by increased myokine secretion by the myotubes as we expected. Others found EPS to significantly increase myokine secretion in human primary muscle cells, but their stimulation protocols were of longer durations[52, 53]. Another important consideration in the interpretation of our results is the discrepancy that was observed by others in the response to EPS treatment as a function of BMI and insulin resistance[64]. Indeed, Park *at al.*, showed that myotubes derived from participants with severe obesity (BMI > 40 kg/m$^2$) and with whole-body insulin resistance showed reduced activation of the AMPK pathway and downstream glucose transporter GLUT4 translocation to the plasma membrane in response to a 24h EPS treatment as compared to myotubes derived from lean and insulin sensitive participants. Since participants in our study livedwith obesity, and some qualified as having severe obesity, there is a possibility that the participants' characteristics impacted the ability of the myotubes derived from their skeletal muscle biopsy to respond to EPS similarly to myotubes isolated from participants not living with obesity. As this study did not include a group of participants without obesity, we cannot determine whether this factor played a role in the observed response to EPS treatment in the assessed myotubes. Also, it is possible that the EPS protocol we employed is sufficient to activate some of the pathways induced during muscle contraction, but that some other metabolic or mechanical adaptations such as $Ca^{2+}$ dynamics are not induced sufficiently to lead to the release of myokines occurring *in vivo* during muscle contraction. That being said, we did observe mechanical contraction of the myotubes at a varying proportion in the majority of the cell lines during EPS exposure (data not shown).

Our analysis of the mRNA expression of myokines in the myotubes obtained from biopsies collected from the participants pre- and post-intervention revealed interesting patterns in their regulation. For example, the expression of pro-inflammatory myokines IL-1β and IL-8 were both upregulated in myotubes from the T2D responder group following the aerobic exercise intervention, whereas IL-15 mRNA was increased post-intervention in the myotubes from the no-T2D and T2D non-responder groups. This finding is counterintuitive, as IL-8 and IL-15 are both found to be increasingly secreted by muscle cells derived from patients with T2D[65, 66]. One hypothesis for increased myokine secretion in human primary myotubes from patients with T2D is that this dysregulation contributes to the low-grade chronic inflammation state observed in this patient population. Therefore, we would expect that increased exercise in patients with T2D would reduce inflammation and downregulate myokine secretion in their muscle. It is possible that we could have observed a downregulation of the expression of these myokines in the patients with T2D at a time-point of collection of the muscle biopsies later than 10 weeks after the start of the exercise intervention or after a longer exercise intervention. Some metabolic and/or signaling changes require a longer period of adaptation and a 10-week intervention is a relatively short snapshot of a chronic metabolic condition ongoing for years prior to the lifestyle intervention. It is also possible that alterations in muscle expression of the myokines are not reflective of protein levels measured in the cell culture supernatant because other post-transcriptional mechanisms affect their accumulation in the medium such as mRNA translation and autocrine/paracrine myokine uptake by the muscle cells.

The most interesting comparison between the response to the aerobic exercise protocol across all groups was the change in expression of IL-10 mRNA. In both the myotubes from the no-T2D and T2D responder groups, IL-10 mRNA was increased post- compared to pre-intervention, while the opposite could be observed in the myotubes from participants in the T2D non-responders group. IL-10 is an anti-inflammatory myokine that positively modulates glucose metabolism[13], and to our knowledge, this is the first exploration of its regulation at the mRNA level in myotubes from patients with obesity with or without T2D accounting for the ability of patients with T2D to respond to exercise training. Although the statistical analysis of IL-10 secretion in the cell culture supernatant of the myotubes showed no significant change in response to the exercise intervention, in all study participants irrespective of group, post-intervention concentrations were higher than pre-intervention. Others also found an increase in IL-10 in the plasma of patients with T2D after an aerobic exercise intervention, but their experimental plan did not include measurement of muscle secretion or expression[67]. Interestingly, Barry *et al.* found what they described as a hyporesponsiveness to the anti-inflammatory effect of IL-10 in patients with T2D[68]. This altered response to IL-10 in the context of T2D might explain the increase in its secretion from muscle cells from patients with this chronic condition to compensate for reduced sensitivity to the anti-inflammatory effect of this myokine. To determine whether the observed increase in IL-10 secretion from the myotubes was similar amongst the groups, we compared the percentage increase in IL-10 concentrations post- compared to pre-intervention and found no significant differences (data not shown).

In summary, contrary to our research hypothesis, we found no definitive implications of myokine secretion in the mechanisms of exercise resistance in patients with T2D. However, we did find novel regulatory patterns of mRNA expression in myotubes derived from participants as a function of the exercise intervention and the ability of patients with T2D to respond to exercise training. We also showed that EPS conditions developed in C2C12 cells to reproduce acute muscle contraction showed great potential as an exercise mimetic in primary human muscle cells, as it activated the AMPK pathway and resulted in visible contraction of the myotubes. Further analyses and observations of the resulting metabolic adaptions in the myotubes must be performed to establish whether this model fully

reproduces *in vivo* muscle contraction. This study highlights that regulation of local myokine signaling in muscle of patients with T2D warrants further investigation to better understand the role of these important signaling molecules in the development of muscle metabolic defects in patients with T2D.

## 4. Materials and Methods

### *4.1. Study Design*

The participant population in this study was included in a larger clinical trial previously published (NCT01911104). Participants had to be sedentary, which corresponds as not physically active ≥3 days per week for a period of 6 months. Samples from 7 participants with obesity (BMI > 30kg/m$^2$) and 13 participants with obesity and T2D (self-reported or with a fasting glucose ≥ 7mmol/L) were used for this secondary analysis. The recruited participants underwent an aerobic exercise training intervention for 10 weeks. Participants living with T2D ceased glucose lowering treatment 15-17 days prior to the start and for the duration of the exercise intervention. Fasted blood samples were collected 72h before the start of the intervention (day -3) and seven days after the last exercise session (day 77). *In vivo* muscle mitochondrial function was assessed via phosphorus ($^{31}$P) magnetic resonance spectroscopy with a 3-T Achieva magnet (Philips Healthcare, Andover, MA) in the partients with T2D at day -3 and 77 to differentiate the responders (increase in PCr recovery rate) from the non-responders (decrease in PCr recovery rate) to the exercise intervention as described previously[42]. Insulin sensitivity (hyperinsulinemic euglycemic clamp), maximal aerobic capacity ($VO_{2peak}$; incremental treadmill test on a Trackmaster TMX 425c (Full Vision, Inc., Newton, KS)) and body composition (dual x-ray absorbsiometry (Lunar iDXA Whole-body Scanner, GE Healthcare Lunar, Madison WI) were also assessed at day -3 and 77. A skeletal muscle biopsy was performed with a Bergström needle in the *vastus lateralis* at day -3 and 77 of the exercise intervention for a sub-group of participants (3 no-T2D, 6 T2D responders, 3 T2D non-responders).

### *4.2. Exercise Intervention Protocol*

The intervention consisted of 4 sessions per week of supervised aerobic exercise on a treadmill following a ramped procotol over 10 weeks[42]. The intensity was monitored as a function of the achieved target heart rate (HR). During weeks 1-4, participants exercised for at least 20 min at an intensity corresponding to 50-70% of their $VO_{2peak}$, increasing to 45 min at the same intensity for weeks 5-8 and for weeks 9-10, participants exercised 45 min at 75% of their $VO_{2peak}$. The adherence to the exercise intervention protocol was measured as the time spent at their target HR during the exercise sessions as a function of the total duration of the sessions.

### *4.3. Cell Culture, Electrical Pulse Stimulation and Preparation of Cell lysates*

Primary human muscle cells were isolated from a fraction of the muscle biopsy and cultured *in vitro*, then purified using the mouse monoclonal 5.1H11 anti-CD56 antibody as previously described[69]. Purified myocytes were grown in Ham-F10 supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone, 10 ng/mL epidermal growth factor, 25 pmol/L insulin (from bovine pancreas, MilliporeSigma, ON, Canada), 1X antibiotic/antimycotic and 5 μg/mL gentamycin (all cell culture reagents were from Wisent, QC, Canada unless otherwise stated) in matrigel-coated (Corning, NY, United-States) T-75 flasks. At passages 4-5, cells were plated in matrigel-coated 6-well plates (Corning, NY, United-States) suitable to fit with the carbon

electrode of the Ion-Optix C-Pace electrical pulse stimulation (EPS) apparatus and grown to confluency before the start of differentiation into myotubes in DMEM low glucose (5 mmol/L; Wisent, QC, Canada) supplemented with 2% FBS, 1X antibiotic/antimycotic and 5 µg/mL gentamycin for seven days. During the last 24h of differentiation, cells were changed to phenol-red free differentiation medium (Wisent, QC, Canada). Myotubes were then treated with three different stimulation conditions for 1h in fresh serum-free phenol-red-free DMEM low-glucose supplemented with 1X antibiotic/antimycotic and 5 µg/mL gentamycin: non-stimulated (NS), electrode exposure (E) (6-well carbon electrode on the cells with no current), and stimulated (S) with 20V 24 ms/sec impulses at 1 Hz as in[58]. The cell culture supernatants were collected, centrifuged at 2000x*g* for 10 min to remove cell debris and kept at -80°C for myokine quantification. The cells were harvested in Trizol® (Invitrogen, MA, US) and kept at -80°C for mRNA isolation. The same EPS protocol was repeated in all cell lines using phenol-red-free differentiation medium (DMEM low glucose with 2% FBS) for immunoblot analyses, with cell lysed in buffer containing 50 mM Tris (pH 8.0), 0.1% Nonidet P-40 (NP-40), 0.5 mM dithiothréitol (DTT), 10 mM sodium fluoride (NaF), 1 mM sodium orthovanadate ($Na_3VO_4$) (all from MilliporeSigma, ON, Canada) and Pierce proteinase inhibitors tablet (Thermofisher, A32963). The lysates were sonicated, centrifuged at 12,000 rpm for 10 min at 4°C and supernatants were stored at -80°C.

*4.4. Immunoblotting*

AMP-activated protein kinase (AMPK) pathway activation was assessed in all cell lines pre- and post-intervention in response to the EPS treatments by polyacrylamide gel electrophoresis. Clarified lysates were prepared from myotubes submitted to EPS treatments in the presence of 2% FBS as described above to prevent the confounding effects of serum starvation on AMPK pathway activation. Cell lysates were separated in Laemmli buffer on 8% SDS-polyacrylamide gels by electrophoresis and transferred unto PVDF membranes for immunoblotting. The primary antibodies used were: P-AMPK$\alpha$ (Thr172) (#2535), AMPK$\alpha$ (#5831), P-ACC (Ser79) (#11818), ACC (#3676), P-AS160 (#4288), AS160 (#2447), diluted 1:500, and α-tubulin (#2144) (Cell Signaling Technologies, MA, United-Sates), diluted 1:1000 all in TBS-Tween 0.1%-BSA 5%. The secondary antibodies used were: anti-rabbit or anti-mouse coupled to horse radish peroxidase diluted 1:5000 in TBS-Tween 0.1%-milk 5% (Santa Cruz Biotechnology, TX, United-States). Proteins were visualised using chemiluminescent substrates on the chemiSOLO (Azure Biosystems) and protein bands were quantified by integrated density analysis in ImageJ2 software (National Institutes of Health).

*4.5. Quantification of Myokine Secretion*

Myokines were measured in serum samples collected from the participants at baseline and follow-up of the exercise intervention, as well as in cell culture supernatants of myotubes derived from biopsy samples obtained at baseline and follow-up timepoints. Myokine quantifications were performed using a single-plex assay (R-plex; SPARC) and a multiplex assay (U-plex; BDNF, FGF21, IL-6, IL-8, IL-10, IL-13, IL-15, and IL-18) from Meso Scale Discovery (Rockville, MD, USA) following the manufacturer's instructions and as previously published[70, 71]. All antibodies with the exception of SPARC were validated for target specificity. More information about specificity tests can be found in the datasheet of the U-plex kits (https://www.mesoscale.com/).

*4.6. Quantification of mRNA Expression*

Cell lysates harvested in Trizol® were processed following the Trizol mRNA isolation protocol. Briefly, chloroform was added to the lysates in a 1:5 (v:v) ratio and samples were vortexed, incubated on ice and centrifuged at 12,000 rpm 15 min at 4°C. The upper (aqueous) phase was then collected and mixed with ice cold isopropanol before centifuging at 12,000 rpm 10 min at 4°C to precipitate the RNA. The pellet was dried and washed in 70% ethanol before storage at -20°C until the day of the assay. On the day of the NanoString assay, samples were thawed on ice, centrifuged at 12,000 rpm 10 min at 4°C and dried completely before resuspending the pellet in RNase-free water. RNA samples were measured with the Nanodrop and concentrations adjusted for downstream analyses. Expression patterns of myokines and other genes of interest was assessed with the nCounter® Human Inflammation V2 Panel from NanoString Technologies. Samples were processed according to the manufacturer's instructions and data were analyzed using the n-Solver Analysis Software V4.0 (NanoString Technologies).

### *4.7. Statistical Analyses*

All patient characteristics were first assessed for normality or lognormality using the D'Agostino & Pearson test. Datasets that followed a lognormal distribution were transformed before further analyses. The age and adherence data were analyzed using an ordinary ANOVA with Šidák's multiple comparison as a post-hoc test. The rest of the patient characteristics pre- (day -3) and post-intervention (day 77) (BMI, HbA1c (%), fasted blood glucose (mg/dl), fat mass (kg), lean mass (kg), glucose infusion rate (GIR) (µmol/kgBW/min), and $VO2_{max}$ (ml/kg/min), as well as the mRNA quantification assays were analyzed by two-way ANOVA with repeated measures or mixed model analysis when some values were missing with Šidák's multiple comparison as a post-hoc test. The quantification data of the immunoblots were analyzed by regular two-way ANOVA with Šidák's multiple comparison as a post-hoc test. Data relating to myokines quantification were first analyzed to identify potential outliers by the ROUT method using a Q = 1%. The 'cleaned' data were then analyzed by two-way ANOVA with repeated measures or mixed model analysis when some values were missing with Šidák's multiple comparison as a post-hoc test. Correlation analysis of myokines measured in cell culture supernatant and in serum samples were performed pooling pre- and post-intervention values from participants across all groups. Data was first assessed for normality using the D'Agostino & Pearson test. Data sets that did not follow a normal distribution were analyzed using the nonparametric Spearman correlation test, whereas those following a normal distribution were assessed using Pearson correlation coefficients. A p-value ≤ 0.05 was considered as significant. Statistical analyses were performed using Prism 10 Software (Graphpad).

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

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# Chapter 5

# General Discussion

## Summary of Findings

We first sought to determine whether acute aerobic exercise could impact the release in circulation of a selection of myokines known to impact muscle metabolism and/or be altered in the context of T2D in subjects with or without obesity. Our study compared the effects of a single session of moderate-intensity cycling (60 minutes at 60% $VO_{2peak}$) on plasma myokine concentrations in six women with obesity and five women without obesity. The inclusion of participants with differing levels of adiposity allowed us to assess whether having obesity could impact circulating levels of these myokines both at basal and in response to the aerobic exercise. We found that obesity was associated with lower plasma levels of IL-8 and SPARC, but higher levels of IL-13. Additionally, FGF21 levels followed a different regulatory pattern between the two groups in the 24h post-exercise, with a more acute increase in the group with obesity compared to the group without. We found that the aerobic exercise bout caused significant variations in plasma levels of IL-6, IL-8, IL-15 and IL-18 over the 24h time-course following exercise. Overall, the study confirmed the influence of exercise on circulating myokines and highlighted the differing responses between women with and without obesity.

Next, we aimed to compare the anti-inflammatory effects of a more traditional training modality (MICT) to a modality gaining in popularity due to its efficacity and tendency to increase protocol adherence (HIIT) in patients with CAD with or without T2D. Low-grade inflammation plays a key role in CAD, as well as T2D, and can be alleviated through exercise. In our secondary analysis of a registered clinical trial, male patients with CAD enrolled in a 12-week cardiac rehabilitation program including either MICT or HIIT were separated according to co-occurrence of T2D.

Cytokines were measured before and after the interventions in plasma samples collected in the resting state to infer inflammatory levels in these individuals. Results showed that patients with both CAD and T2D had higher levels of plasma IL-8. The training interventions resulted in changes in plasma levels of FGF21 and IL-6, with greater reductions observed in patients with both CAD and T2D. HIIT caused an increase in circulating SPARC in participants without T2D but a decrease in those with T2D, while the opposite was true for MICT. Both training modalities significantly reduced circulating levels of several cytokines, including FGF21, IL-6, IL-8, IL-10, and IL-18, regardless of T2D status. In conclusion, both HIIT and MICT effectively reduced plasma concentrations of cytokines associated with low-grade inflammation in patients with CAD, with more pronounced effects on FGF21 and IL-6 in patients with T2D. Contrary to our initial hypothesis, there were no differences in anti-inflammatory potential between MICT and HIIT protocols, albeit HIIT yielding similar results to MICT with lower training volume.

Our last study set out to observe both the peripheral and local regulation of myokine secretion in a population of individuals with obesity with or without T2D, while accounting for the ability of patients with T2D to respond to the beneficial effects of exercise. As mentioned previously, on average exercise is an efficient method for the management of symptoms of T2D, but for some patients training interventions do not result in improvements of glucose homeostasis. Participants with or without T2D engaged in a standard 10-week aerobic exercise program designed for the management of T2D symptoms, and those with T2D were further classified as responders or non-responders based on improvements in muscle mitochondrial function following the intervention. Myokine concentrations were measured in serum and cell culture supernatant of biopsy-derived hSkMCs collected from the participants pre- and post-intervention, along with protein concentrations and gene expression in the cell lysates. Overall, there were no significant

differences in myokine secretion based on T2D status or the ability to respond to exercise, except minor effects detected for certain myokines (IL-1β, IL-8, IL-10, and IL-15). Moreover, we were unable to validate an *in vitro* model of acute muscle contraction developed in a murine muscle cell line using hSkMCs. In our hands, visible contraction of a small proportion of myotubes could be observed, as mirrored by the significant induction of AMPK pathway activation in cell lines of some of the groups of participants, although not all. Conversely, an increase in secretion of myokines thought to be induced by muscle contraction *in vivo* did not accompany this activation of the AMPK pathway. Our findings suggest that while myokine secretion may not significantly differ with exercise response in T2D, further research is needed to understand the molecular mechanisms at play and their potential impact on exercise adaptations in this population.

A summary of our findings across all three exercise studies can be found on the next page (Supplementary Table X).

*Supplementary Table 1. Summary of results in myokine variations across studies.*

| Myokine | Acute exercise | 12-week exercise intervention | 10-week exercise intervention | | |
|---|---|---|---|---|---|
| | Plasma | Plasma | Serum | Cell secretion | Cell expression (mRNA) |
| **TNF-α** | - | | - | - | |
| **IL-1β** | - | | - | - | ▲ |
| **IL-6** | ▲ | ▼ | | | |
| **IL-10** | | ▼ | | | ▲ |
| **IL-18** | ▲ | ▼ | | | |
| **IL-8** | ▼ ▼ | ▼ ▼ | ▲ | | ▲ |
| **IL-13** | ▲ | | | | |
| **IL-15** | ▲ | | | | ▲ |
| **SPARC** | ▼ | | | | - |
| **FGF21** | ▲ | ▼ | | | - |

Regulation of myokines is portrayed with arrows pointing upwards (increase) or downwards (decrease) as a function of exercise (black), obesity (teal) or T2D (pink). Acute exercise refers to study objective 1 (chapter 2), 12-week exercise intervention refers to study objective 2 (chapter 3) and 10-week exercise intervention refers to study objective 3 (chapter 4).

### The Search for the Exercise Factor

Using dogs, M.S. Goldstein instilled curiosity and determination in the exercise physiology scientific community by showing that transfer of lymphatic fluid derived from the contracting limb of an animal to another animal in the resting state could induce glucose uptake as portrayed by reduced glycemia[211]. This effect was maintained in de-pancreatized dogs used as a model of T2D, suggesting that the unknown factor(s) could be used an alternative management method to performing exercise in this patient population. Since then, a lot of work has gone to pin-point the source of the factor(s) released in circulation that could mediate the beneficial effects of exercise on whole-body glucose metabolism. Some speculated that myokines that could fulfill this role would be increased in the plasma following acute exercise in order to transduce increased glucose uptake in peripheral tissues via endocrine signaling[212]. Using this logic, they suggested IL-6, SPARC, ANGPLT4, chemokine (C-X3-C motif) ligand 1, and chemokine (C-C motif) ligand 2 were most likely to act as exercise factors. Although we did not measure the last three targets in the study samples, we were unable to detect a regulatory pattern in plasma SPARC concentrations following an aerobic exercise bout in women with or without obesity. On the other hand, FGF21, IL-6, IL-8, IL-15 and IL-18 all showed transiently increased levels in the circulation with varying timing of release and resorption over the 24h following exercise. Our findings suggest that these myokines have the potential to mediate the acute beneficial effects of exercise in increasing glucose uptake, as some were increased immediately after the effort (*i.e.* FGF21, IL-6, IL-15) and have previously been shown to contribute to skeletal muscle glucose uptake[62,213,214]. As our *in vitro* model of muscle contraction (chapter 4) did not yield clear increases in the levels of these myokines, it is not possible to draw correlative conclusions on the source of these myokines in

plasma following acute exercise. However, all three candidates have been shown to be increased in muscle or increasingly released by muscle cells during contraction in other studies[46]. Interestingly, data from an unpublished study of my PhD showed that unidentified factors from the secretome of biopsy-derived hSkMCs from participants with a healthy BMI submitted to EPS induced increased glucose uptake in un-stimulated healthy hSkMCs from a different individual (Appendix 1, Figure S1). These preliminary *in vitro* findings further support the hypothesis by which factors originating from muscle during contraction could favor glucose uptake in non-exercising muscle, although the nature of these factors remains unsolved.

## The Involvement of Myokines in Chronic Low-grade Inflammation

As many different researchers brought forward at the 2024 Cell Symposia on Exercise Metabolism, many chronic non-communicable diseases show a central chronic inflammation component that can be improved through exercise-based therapy. The anti-inflammatory effects of exercise training is widely agreed upon[215], and we were able to show that this applies to circulating levels of candidate myokines in the plasma of participants with CAD with or without T2D following two different exercise interventions (published manuscript presented in chapter 3), but not in the serum of a participant population including people with obesity with or without T2D following aerobic exercise training (published manuscript presented in chapter 4). This discrepancy is in alignment with the fact that most myokines are better seen to be regulated by exercise in plasma than serum samples[216]. Nonetheless, we observed a significant effect of training interventions in reducing plasma levels of a number of cytokines that can also be characterized as myokines in the patients with CAD, for which the effect was greater in patients with both CAD and T2D when considering the levels of IL-6 and FGF21. These results build on the findings of others suggesting that reductions in plasma IL-8 and MCP-1 in relation to decreased visceral

adiposity following exercise training could serve as markers of reduced inflammation in subjects with metabolic syndrome to prevent both CAD and T2D onset[217]. It would be interesting to delve deeper into the potential of IL-6 and FGF21 as similar markers of the risk of development and/or disease severity in regards to CAD and T2D.

A contribution of skeletal muscle to chronic inflammation has been hypothesized upon the observation that biopsy-derived hSkMCs from patients with T2D cultured *in vitro* showed intrinsically altered myokine secretion[86]. In the cell lines from the published study presented in chapter 4, we were unable to replicate these findings for the myokines we measured, even if some targets had been shown by Ciaraldi *et al.* to be increasingly secreted (*i.e.* IL-6, IL-8 and IL-15) in the context of T2D. That said, as discussed previously, we measured the myokines in medium exposed to the hSkMCs for a duration of 1h, whereas they had quantified the myokines 24h after exposure to the cells. In support of this factor being a limitation to the detection of significant differences, data from the unpublished study conducted during my PhD comparing myokine secretion in two healthy cell lines and five cell lines derived from patients with obesity and T2D showed distinct secretory patterns between groups (Appendix I, Figure S2). For this study, the medium was exposed to the cells for 24h and almost all myokines measured in the cell culture medium were increased in the cell lines derived from participants with T2D (*i.e.* BDNF, IL-6, IL-8, IL-10, IL-13, IL-15, IL-18), with the exception of FGF21 levels that seemed higher in the cell lines derived from healthy participants. Since we did not have data in individuals with obesity without T2D for comparison in this study yet, and that individuals with T2D in the Ciaraldi et al. study had a significantly higher BMI than individuals without T2D[86], it is impossible to conclude that these alterations in myokine secretion are due to T2D status, and not obesity.

Supporting the results from the study presented in chapter 4, EPS did not result in a clear increase in myokine secretion in either cell lines derived from subjects with or without T2D in the unpublished study (Appendix I, Figure S2), even when using prolonged stimulation conditions lasting up to 24h with a lower voltage but otherwise similar impulse trains (11.5V, 24ms/s and 1Hz frequency). Some mechanistic structures and metabolic aspects crucial to molecular signaling cascades regulating energetic adaptations to contraction in the hSkMCs differentiated into myotubes *in vitro* cannot be attained using conventional cell culture methods due to a number of factors including the limited 3D structure of the myotubes. For example, the cells show low contractility[184,218], as observed in our studies, the insulin-induced increase in glucose uptake is diminished, likely owing to a disproportionate GLUT4/GLUT1 transporter ratio compared to *in vivo*[219,220] and they show more glycolytic properties rather than oxidative for the production of ATP[221]. To improve this model of exercise in a dish, Dreher *et al.* recently fine-tuned the differentiation conditions of the myotubes by using serum-free medium and supplementing with insulin growth factor (IGF)-1 at a concentration matching physiological plasma levels[222]. In doing so, they were able to develop human myotubes with a rescued version of all limitations described above over 10 days of differentiation. Although 3D culture models have also been published previously[223,224], this model is readily accessible and seems to mimic *in vivo* skeletal muscle attributes faithfully. In future studies of acute muscle contraction using biopsy-derived hSkMCs from patients with T2D, it would be interesting to test whether these differentiation conditions yield myotubes of similar quality as with healthy donors and then compare myokine secretion between the two groups.

## Study Limitations

One of the most important limitations across all three studies included in this dissertation is the absence of groups not performing either the acute or chronic exercise prescriptions for comparison. As highlighted in all three discussions of the published articles, the lack on an appropriate control group not performing the exercise prevents the exclusion of the possibility that all variations in levels of myokines observed are the product of time-elapsed and not the exercise itself. In the case of acute exercise, circulating myokine levels could fluctuate throughout the day as a function of natural circadian rhythms. Similarly, when considering chronic exercise or training interventions, the natural evolution of physiological conditions (overweight, obesity, CAD, T2D) over time could impact circulating levels of myokines in the participant populations.

Another limiting factor in the interpretation of our study results is the absence of proteomics analyses, restricting the number of target myokines for which observations can be made. To this day, some myokines remain hard to quantify with specificity and precision without having to resort to mass spectrometry analysis of samples, as commercially available ELISA kits are rare and/or can target non-active forms of the peptide of interest[143,225,226]. We were still able to assay a number of candidate myokines within our studies and to perform a high-throughput analysis of mRNA transcripts in the cell lysates of the chapter 4 clinical cell lines, which showed the absence of clustering amongst the groups.

Finally, an important aspect of skeletal muscle signaling that was not measured in the course of these studies is the possibility of myokines being secreted within or induced by the cargo of EVs. In other chronic non-communicable diseases such as arthritis, cytokines can be upregulated or downregulated by local release of EVs containing miRNAs that exacerbate inflammation in the musculoskeletal joint[179,180]. A similar mechanism could be envisioned in the skeletal muscle

interstitial space during muscle contraction, upregulating myokines with a positive effect on muscle metabolism through miRNAs contained in EVs and released locally.

## Future Directions

Complimentary to our observations of the effects of exercise in patients with T2D on myokine secretion in the circulation and local release by muscle cells *in vitro*, it would be interesting to delve deeper into the autocrine/paracrine role of myokines in regulating muscle metabolism in this patient population. In the course of my PhD, I was able to perform preliminary assessments of the metabolic effects resulting from exposure of hSkMCs derived from participants with a healthy BMI to the secretome of hSkMCs derived from subjects with obesity and T2D. This unpublished study included the quantification of insulin-dependent glucose uptake following 24h exposure of the healthy myotubes to the conditioned medium of myotubes from patients with T2D (Appendix I, Figure S3). On the whole, we saw a decrease in the insulin-induced fold increase in glucose uptake in the healthy myotubes upon exposure to the secretome of myotubes derived from patients with T2D that varied between individual cell lines, suggesting that factors in the T2D secretome were sufficient to impair insulin sensitivity in the healthy hSkMCs. The interindividual variability could be explained, in part, by the participant characteristics, as correlation analyses between the fold-increase in glucose uptake with insulin compared to basal and the HbA1c values of the corresponding participants were significantly linked. A negative correlation ($p=0.04113$, $r=-0.5953$) could be observed when plotting both sets of values together, indicative of a potential effect of poor glucose homeostasis in the participants concomitant with a negative effect of their secretome on insulin sensitivity in the healthy myotubes. These preliminary data support a previous study from my PhD supervisor showing the negative effect of unknown factors contained in the secretome of hSkMCs from patients with T2D on the abnormal localization of a fatty acid

transporter to the plasma membrane in healthy hSkMCs[177]. A more profound examination of the potential autocrine/paracrine effect of the secretome from hSkMCs derived from patients with T2D should be undertaken to better characterize the putative role of myokines and/or other muscle secreted factors in the development of muscle metabolic defects.

Intensive lifestyle interventions combining changes in nutrition and habitual physical activity have proven to be effective for the management of glucose homeostasis in patients with obesity and T2D, sometimes allowing for remission from this disease and sustained improvements in body composition[227,228]. However, these types of interventions are not accessible to everyone living with T2D (remote regions, limited access to healthcare providers, limited knowledge of nutrition and physical activity practices, cultural barriers, etc.). The study of myokine secretion in individuals who practice habitual physical activity in an unstructured manner (*i.e.* frequent and abundant walking, active commuting) could uncover interesting findings in comparison to the regulation of myokine secretion following structured physical activity, which represents the bulk of information currently available in the literature. It is possible that the secretion of myokines in sedentary individuals who incorporate physical activity for a shorter period of time does not reflect the secretion of myokines in people who have been active for a longer period of time. We might be missing important regulatory patterns due to the scope of participants included in exercise studies when measuring myokines secretion in circulation or muscle-derived samples. Also, since exercise training interventions cannot be prescribed to all patients with T2D for the management of their symptoms or the reduction of co-morbidity incidence (*e.g.* CAD) a better understanding of this alternate type of lifestyle modifications and their impact on glucose homeostasis and muscle signaling events could open new and more inclusive avenues of therapeutic recommendations.

## Conclusion

We were able to characterize the effects of both acute and chronic exercise on the regulation of myokine release in the circulation and their secretion by muscle specifically in various populations of subjects (overweight, living with obesity, patients with T2D qualifying as responders or non-responders to exercise training, patients with CAD and T2D). Our studies contribute to the literature detailing the molecular mechanisms instigated by exercise in the context of T2D and related co-morbidities. The findings presented in this thesis advance the field of study of myokines as potential diagnostic and therapeutic targets for the management of T2D, specifically in the context of exercise as an avenue of treatment for this disease.

Every aspect of the research presented herein was performed as a collaborative effort with various teams in the Ottawa region and beyond. Academic research is a field in which collaboration is key and my PhD thesis highlights this fact, since we were able to consolidate data from four distinct clinical studies to draw observations and achieve our research aims.

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Appendix I

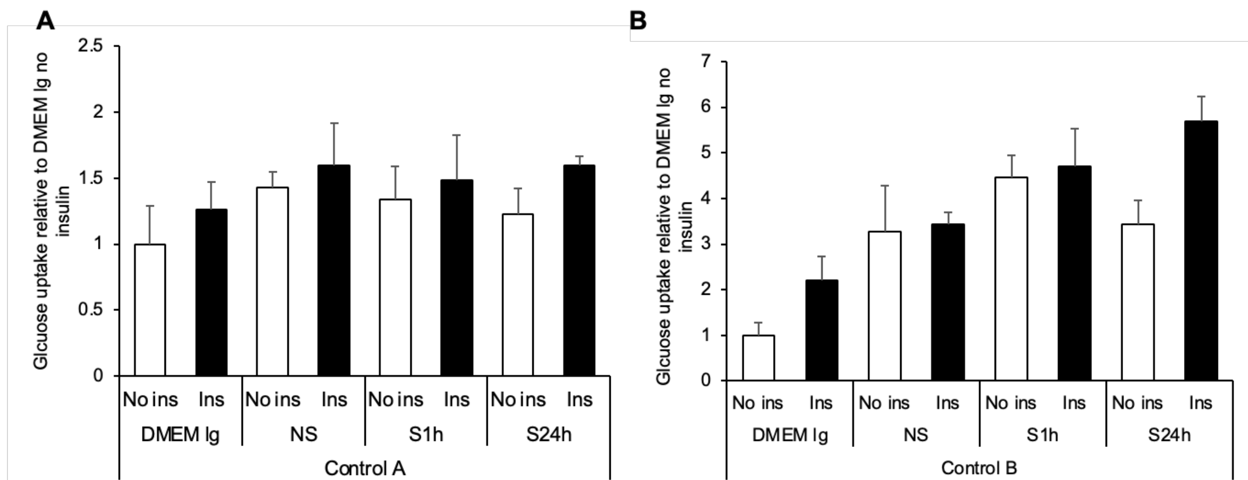


*Figure S1. The effect of exposure to CM obtained from stimulation of healthy myotubes on glucose uptake in these myotubes. Primary muscle cells from two healthy participants (control A and B) were grown to confluency in 6-well plates and differentiated for 6 days. During the last 24h of differentiation, the myotubes were serum starved in DMEM low glucose (lg) free of phenol red and treated with no EPS (NS), 1h of EPS (20V , 24ms/s, 1Hz; S1h) or 24h of EPS (11.5V , 24ms/s, 1Hz; S24h). The cell culture supernatant was collected as CM and used to treat myotubes from the same cell lines in a cross-over design for 24h on day 6 of their differentiation in 48-well plates, with control wells treated with DMEM lg phenol red-free. At the end of the 24h, half of the wells were treated with 100nM of insulin and glucose uptake was measured with 0.5 µCi/ml 3H 2-deoxy-glucose and 10µM 2-deoxy-glucose and counted via liquid scintillation (Tri-Carb 4190, Perkin Elmer). Cytochalasin B (10µM) was used to control for non-specific glucose uptake. Data are presented relative to DMEM low glucose ± SD for the different wells in the same conditions. N=2 individual experiments presented as (A) Control cell line A treated with the CM of myotubes from participant B and (B) Control cell line B treated with the CM of myotubes from participant A.*

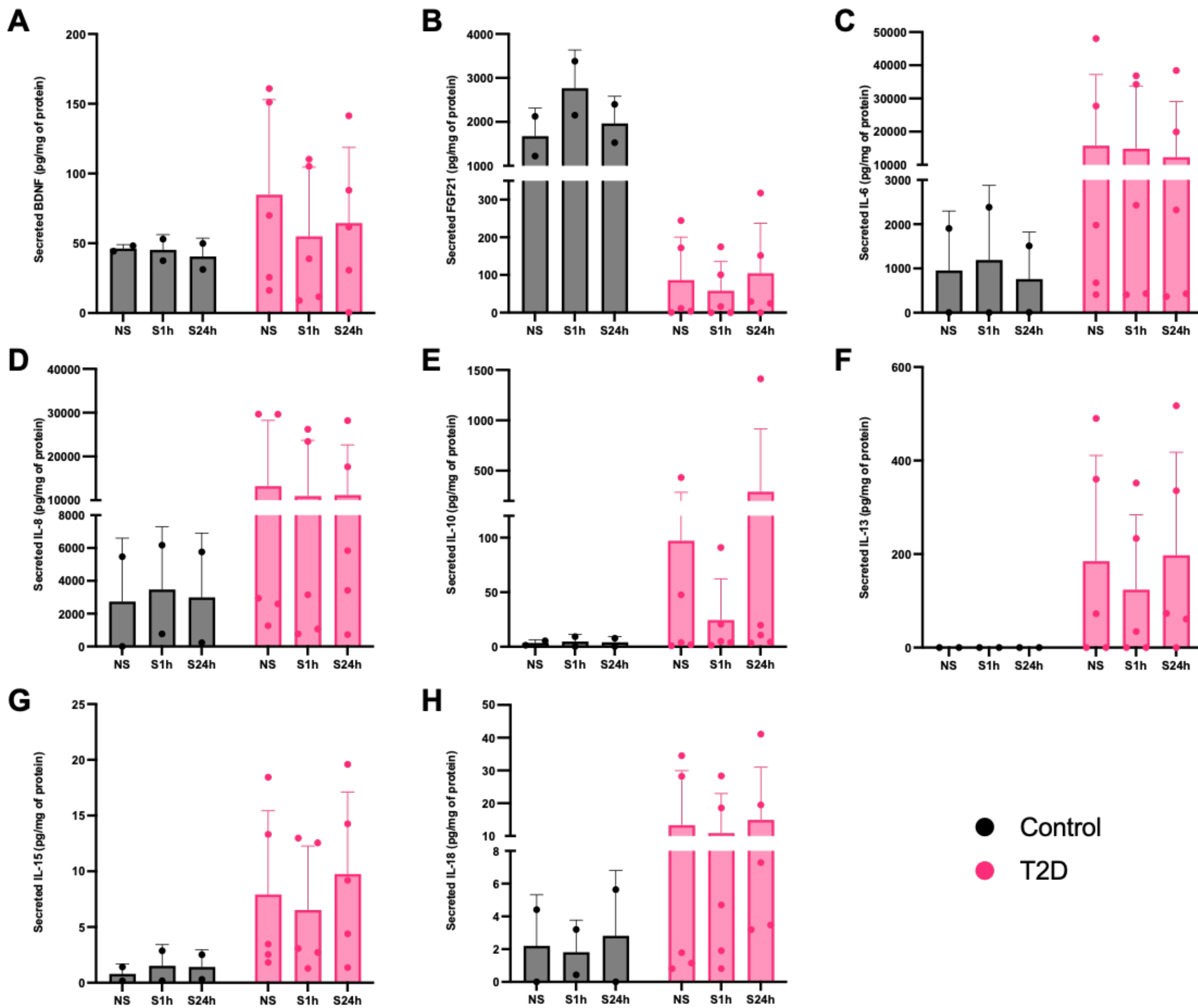


*Figure S2. Quantification of myokine secretion from myotubes of healthy participants and patients with T2D in response to contraction. Primary muscle cells from healthy participants (control group, n=2) and patients with T2D (T2D group, n=5) were grown to confluency in 6-well plates and differentiated for 6 days. During the last 24h of differentiation, the myotubes were serum starved in DMEM low glucose (lg) free of phenol red and treated with no EPS (NS), 1h of EPS (20V , 24ms/s, 1Hz; S1h) or 24h of EPS (11.5V , 24ms/s, 1Hz; S24h). The cell culture supernatant was collected to measure candidate myokines via multiplex assay (U-plex, Meso Scale Discovery) and protein content in cell lysates was quantified via DC protein assay (BioRad). Quantification of (A) BDNF, (B) FGF21, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) IL-15, and (G) IL-18 secretion normalized to protein content in cell lysates. Individual data points are shown, with mean ± SD. Black dots = control group, pink dots = T2D group.*

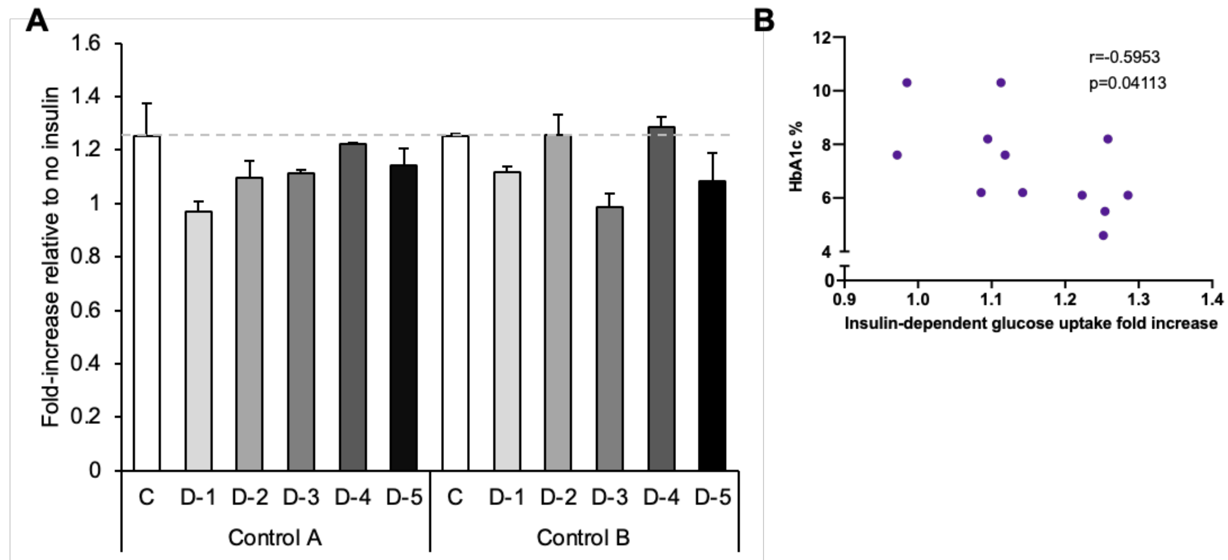


*Figure S3. Effect of exposure of healthy myotubes to the CM of myotubes from patients with T2D on glucose uptake with or without insulin treatment. (A) Primary human muscle cells from two healthy participants (control A and B) were differentiated 7 days in 48-well plates to obtain myotubes and were treated with CM from myotubes of their own cell line (control; C) or from patients with T2D (D-1-5) for the last 24h of differentiation, and stimulated with 100nM insulin for 20 minutes. Glucose uptake was measured with 0.5 µCi/ml 3H 2-deoxy-glucose and 10µM 2-deoxy-glucose and counted via liquid scintillation (Tri-Carb 4190, Perkin Elmer). Cytochalasin B (10µM) was used to control for non-specific glucose uptake. Data are presented as fold-increase relative to no insulin treatment ± SD of technical triplicates. The gray dotted line indicates the fold-increase with CM treatments from the healthy myotubes for reference. (B) Relationship between glucose uptake fold-increase in response to insulin and the HbA1c (%) values of the participant from whom the myotubes to prepare the CM are derived. Each data point represents one CM condition (n=1 healthy control and n=5 patients with T2D for each control cell line; n=12 total).*

# Appendix II

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