Identifying Salivary Biomarkers Associated with Prolonged Sitting

by

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THESIS EXAMINATION INFORMATION

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An oral defense of this thesis took place on April 18, 2023, in front of the following examining committee:

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The above committee determined that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate during an oral examination. A signed copy of the Certificate of Approval is available from the School of Graduate and Postdoctoral Studies.

ABSTRACT

The effect of prolonged sitting on salivary biomarker concentrations is relatively unexplored. The purpose of this work was to identify novel biomarkers, detectable in the saliva in response to prolonged and interrupted bouts of sitting.

Participants completed three sessions. The first session included a baseline saliva sample and maximal exercise test. The remaining two sessions were performed in random order. One, a prolonged sitting session (4-hours of sitting), and the other, an interrupted sitting session (4-hours of sitting with 3-minute moderate-intensity walking interruptions every 27 minutes). Saliva samples were collected pre- and post-session. A human cytokine antibody array was then used to detect biomarkers present in the saliva.

A total of 26 biomarkers were detectable in the saliva of young, healthy males and females. Prolonged and interrupted sitting lead to changes in salivary biomarker concentrations, with variations dependent on sex.

Keywords: sedentary behaviour; biomarkers; cytokines; growth factors; inflammation

AUTHOR'S DECLARATION

I hereby declare that this thesis consists of original work of which I have authored. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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______________________________ Emmeline Meens Miller

STATEMENT OF CONTRIBUTIONS

The manuscript included in Chapter 5 was performed in the Human Health and Performance Laboratory at the University of Ontario Institute of Technology and will be submitted for publication. Co-authors of the manuscript include Dr. Shilpa Dogra, Dr. Julia Green-Johnson, Dr. Michael Jeffrey, and Nicholas O'Rourke, MHSc. Investigation and data collected was completed by me with the assistance of Nicholas O'Rourke and laboratory analysis was completed with the assistance of Dr. Michael Jeffrey. Reviewing, editing, and supervision was provided by Dr. Shilpa Dogra, Dr. Julia Green-Johnson, and Dr. Michael Jeffrey, as I, the first author, performed all data synthesis, primary interpretations, and writing of results. I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication. I have used standard referencing practices to acknowledge ideas, research techniques, or other materials that belong to others.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMR	Antimicrobial-resistant
ANG	Angiogenin
AU	Absorbance Units
BAF	B-cell Activating Factor
bFGF	basic fibroblast growth factor
BMI	Body Mass Index
BSF	B-cell Stimulatory Factor
C	Celsius
C3	Complement Component 3
CASP	Caspase
CCL	Chemokine Ligand
CDF	CTL Differentiation Factor
CLMF	Cytotoxic Lymphocyte Maturation Factor
CO2	Carbon Dioxide
CRP	C-Reactive Protein
CSF	Colony-Stimulating Factor
CSIF	Human Cytokine Synthesis Inhibitory Factor
CXCL	Chemokine (C-X-C motif) Ligand
DNA	Deoxyribonucleic Acid
EDF	Eosinophil Differentiation Factor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ENA	Epithelial Neutrophil-Activating Peptide
FAF	Fibroblast-Activating Factor
FMD	Flow-Mediated Dilation
GCF	Gingival Crevicular Fluids

GCSF Granulocyte-Colony Stimulating Factor

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

GRO Growth Regulated Protein/ Growth-Regulated Oncogene

HELENA Health Lifestyle in Europe by Nutrition in Adolescence

HIIT High Intensity Interval Training

HR Heart Rate

HRP Horseradish Peroxidase

hsCRP High Sensitive C-reactive Protein (hsCRP)

IFN Interferon

IGF Insulin-like Growth Factor

IL Interleukin

IP Gamma-Induced Protein

IS Interrupted Sitting

KL KIT-Ligand

LAF Lymphocyte-Activating Factor

LKN Leukoactin

LP Lymphopoietin

MAST2 Microtubule Associated Serine/Threonine Kinase 2

MCGF Mast Cell Growth Factor

MCP Monocyte Chemoattractant Protein

MCSF Macrophage Colony Stimulating Factor

MDC Macrophage Derived Chemokine

MET Metabolic Equivalent Units

MGDF Megakaryocyte Growth and Development Factor

MGIF Melanoma Growth Inhibition Factor

MGSA Melanoma Growth Stimulating Activity

MICT Moderate Intensity Continuous Training

MIG Monokine Induced by Interferon Gamma

mRNA Messenger Ribonucleic Acid

miRNA Micro Ribonucleic Acid

MVPA Moderate-Vigourous Physical Activity

NAP Neutrophil-Activating Protein

NKSF NK Cell Stimulatory Factor Chain

O2 Oxygen

OSM Oncostatin M

PDGF Platelet-Derived Growth Factor

PMT Photomultiplier Tubes

PS Prolonged Sitting

RANTES Regulated upon Activation, Normal T Cell Expressed and Secreted (CCL5)

RER Respiratory Exchange Ratio

RFU Relative Fluorescence Units

RLU Relative Light Units

RNA Ribonucleic Acid

RT-PCR Reverse Transcription Polymerase Chain Reaction

SCF Stem Cell Factor

SD Standard Deviation

SDF Stromal Cell-Derived Factor

SIT Sprint Interval Training

sFlt-1 Soluble VEGF Receptor-1

T1 Time 1

T2 Time 2

TARC Thymus and Activation Regulated Chemokine

TGF Transforming Growth Factor

THPO Thrombopoietin

TNF Tumour Necrosis Factor

TRF T-cell Replacing Factor

VEGF Vascular Endothelial Growth Factor

VO2 Maximal Oxygen Consumption

VPF Vascular Permeability Factor

WBC White Blood Cells

WMF Whole Mouth Fluid

WMS Whole Mouth Saliva

1. Thesis Introduction

1.1. Introduction

Canadians are engaging in large volumes of sitting time. Current data indicates that the average Canadian is spending nearly 40% of their day sitting [1]. This increase in time spent sitting can been attributed to increased sedentary pursuits including commuting, occupations, education, technological advances, leisure time, and the effects of the COVID-19 pandemic [2-6]. Sitting time has been associated with worse health outcomes and all-cause mortality; data indicates that sitting time is associated with impaired vascular function [7], that is, alterations in vascular, growth, and cardiometabolic risk factors [8, 9], changes in blood pressure [10], impaired blood flow [11], as well as a poor metabolic profile [12], that is, increased risk of obesity and diabetes, a decrease in insulin sensitivity and glucose tolerance, and poor metabolic health outcomes [13]. There is some evidence to suggest that excessive sitting is also associated with higher levels of chronic low-grade inflammation [14]. Novel research using pro-inflammatory and anti-inflammatory biomarkers supports this association [15, 16]. However, to date, the effect of sitting time on inflammatory markers and vascular and growth factors has been understudied. Furthermore, studies that have examined this association have focused on a limited number of biomarkers.

My thesis aims to address two major gaps in our understanding of prolonged sitting and human physiology. First, to identify cytokines, vascular factors, and growth factors that respond to bouts of prolonged and interrupted sitting. Second, to determine if there are sex differences in which biomarkers respond to prolonged and interrupted sitting.

By identifying ***novel biomarkers*** that respond to prolonged bouts of sitting, this research can help elucidate the underlying physiological mechanisms linking sedentary time to physiological disruptions (e.g., vascular dysfunction) and ultimately, poor health outcomes. By determining if there are sex-differences in the response of various biomarkers, this work will contribute to our understanding of physiological differences between males and females, providing insight into therapeutic targets for different health conditions. Finally, by using saliva to analyze biomarkers, this work may contribute to

methodological advancements, enabling new areas of research using a non-invasive method.

Research Questions

1. Which salivary biomarkers respond to prolonged and interrupted sitting in young, healthy males and females?
2. Are there sex differences in which biomarkers respond to prolonged and interrupted sitting?

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2. Literature Review

2.1. Sedentarism

2.1.1. Terminology

Sedentary behaviour was recently defined by a consensus exercise undertaken by international leaders in the field, as any waking behaviour that has an energy expenditure of less than or equal to (≤) 1.5 Metabolic Equivalent Units (METs), while in a sitting, reclining, or lying posture [1]. Sedentary behaviours include leisure, occupational, household, and transportation activities, excluding sleeping [2]. **Sedentary time** is the total time spent engaging in sedentary behaviours [1]. This refers to the total time for any duration and in any context of seated, reclined, or lying postures. **Sitting time** is a sub-category of sedentary time, referring to only time spent seated, and is a common measure for sedentary time [3]. For example, an office worker may spend several hours per day sitting but spend two hours lying down watching TV in the evenings. The latter would not be captured as "sitting" time. **Sedentarism** is a broad term that can be used to capture these terms.

The periods of time spent engaging in sedentary behaviour are referred to as bouts. **Sedentary bouts** are defined as periods of uninterrupted sedentary time [1], such as sitting, reclining, or lying, without a break and can also be referenced as periods of **prolonged sedentary time**. Prolonged periods of sedentary time can be interrupted with **sedentary interruptions/breaks**, whereby sedentary bouts are disrupted with periods of standing, walking, stretching, and/or exercise. **Sedentary interruptions/breaks** are bouts of non-sedentary time between two sedentary bouts [1].

On the other hand, **physical inactivity** is an insufficient activity level to meet the current physical activity recommendations [1] and is distinct from sedentary time. The current Canadian physical activity guidelines, 24-hour movement guidelines for individuals 18-64 years of age, recommend 150-minutes of moderate-to-vigorous aerobic physical activity per week, muscle strengthening activities using major muscles groups at least twice per week, and several hours of light physical activity, such as standing [4]. Individuals can be considered active yet sedentary, when they meet or exceed the physical

activity guidelines for their demographic but spend large quantities of time sedentary. For example, an individual may meet the recommended guidelines for physical activity by going for a daily 30-minute walk, but then spend the remainder of their day sedentary at their desk or in front of the television. Alternatively, an individual may be considered inactive and sedentary, when they fail to meet the physical activity guidelines and are spending large volumes of time sedentary [5]. In both cases the individual engages in large volumes of sedentary time.

2.1.2. The problem: Too much sedentary time

The prevalence of sedentarism is high, with Canadians spending 69% of their waking hours engaging in sedentary pursuits [6]. There has been a significant increase in sedentary time over the past 50 years [7], with more individuals engaging in this behaviour and simultaneously engaging in less physical activity. Data indicates that the average Canadian is spending 9.5 hours of their waking day sitting, while only 17% of Canadians are meeting the aforementioned 24-hour movement guidelines [8-10]. The following section will address some of the ways in which sedentarism is increasing due to lifestyle and behaviour changes.

Increasing sedentary time is apparent within ***all age groups***; there has been a significant increase in sedentarism from youth to young adult age groups as screen time increases, leading to the negative long-term effects of increased sitting [5]. Despite screen time playing a large role in the increase in sedentarism, older adults are spending a greater amount of time sitting compared to all other age groups [8], this is especially important as this population is already at increased risk for the negative long-term effects associated with increased sitting due to age-related declines. A change from physically demanding occupations and active lifestyles to a more sedentary lifestyle has contributed to increased sedentarism; transportation modes and increased use of technology have also contributed to the increase in sedentarism [11].

The increased ***use of technology*** and ultimately higher levels of screen time have led to an increase in the amount of time spent sitting. For example, among occupations that require a stronger online presence or rely on online communication more than in person communication and interaction, there has been an increase in sedentary and screen time.

Screen time has also risen due to increased technology dependent leisure time and improved access to technology [12-14]. According to a study by Bucksch et al., screen time has increased in adolescents by nearly 2 hours from 2002-2010 [15]. Tremblay et al., found that occupations require a greater use of technology, that children are spending more time using technology, and that easier access to technology has resulted in increased use and therefore more sedentary behaviour [1]. An important point to note is that technology use and screen time can be, and have been, used as a proxy measure for sedentary behaviour, for example, using self-reported screen time to quantify an individual's sedentary time.

Occupational requirements have transitioned to match the digital age. An increased reliance on digital means of conducting business, including sales and communication, has resulted in a higher volume of sedentary time while at work. In their 2011 study, Church and colleagues found that there has been a progressive increase in the popularity of sedentary or minimal physical activity occupations since 1960, they also state that the incidence of moderate intensity physical activity occupations has decreased from 48% to 20% from 1960 to 2008 [11]. This shift from occupations that require physical activity, to occupations that require minimal to no physical activity, has resulted in larger amounts of sedentary time within the population [11].

Transportation methods have also changed dramatically leading to higher volumes of sedentary time. With the development of suburbs and easier access to personal transportation, many people have moved away from city centres. This urban sprawl has led to longer commuting times and therefore greater volumes of sedentary time as well as a reduction of physical activity, as people use passive methods of transport. Alternately, active commuting or active transportation methods have been associated with increased levels of physical activity and lower volumes of sedentary time [16].

Summary: Sedentarism is a broad term that refers to sedentary time, behaviour, and bouts. People of all ages are accumulating large volumes of sedentary time.

2.2. Sedentary Physiology

2.2.1. What we know about sedentary physiology

While the physiological response to exercise, or a lack thereof, has been well studied over the past several decades, research on the physiological effects of sedentarism have only recently been the focus of researcher's attention. As stated by Tremblay et al., the science of sedentary behaviour requires further investigation as a complementary and distinct area of research, as this could inform the physiological and biological responses to movement and the prevention of chronic disease to support policy making and improve population health [17]. As sedentary behaviour becomes more prevalent within our population, understanding the physiological changes associated with sitting becomes more important.

Below is a review of literature on the vascular and metabolic response to prolonged sitting.

2.2.2. Vascular function

Evidence suggests that prolonged sitting (1-6 hours) impairs vascular function [18], specifically within the lower limb, and the femoral and popliteal arteries [18, 19]. Endothelial function maintains vascular tone and blood fluidity; it can be measured using non-invasive techniques such as flow-mediated dilation (FMD), calf circumference, reactive hyperemia index using pulse amplitude tonometry, and ultrasound [19]. Prolonged bouts of sitting have been associated with higher blood pressure, impaired blood flow, reduced venous muscle pump and venous return, and higher levels of systemic inflammation [20-22]. Though there is a clear effect of sedentarism on vascular function, the specific mechanisms through which sedentary behaviour is linked with vascular dysfunction are unknown. In the following section, observational and experimental studies investigating the link between sedentary behaviour and vascular dysfunction are reviewed.

Population based studies provide several insights into the association between sitting and vascular dysfunction. For example, an analysis of the Multi-Ethnic Study of Atherosclerosis (n = 1543) showed that sedentary behaviour was associated with higher levels of Leptin and Tumour Necrosis Factor Alpha (TNF-α), but lower adiponectin-to-leptin ratios, which are indicators of adiposity-associated inflammation [23]. Similarly, several studies have shown that sitting is associated with higher blood pressure [24, 25],

such that those who engage in high levels of sitting have higher systolic and diastolic blood pressure. Those who engage in more sedentary behaviour and less physical activity are also more likely to have an increased risk of cardiovascular and all-cause mortality [26, 27]. Vascular function measured using pulse amplitude tonometry has also shown an association with sedentary time [28]. Clearly there is observational evidence to support an association between sedentary behaviour and vascular dysfunction; this has begun to be investigated in experimental research, where the exact dose-response can be investigated.

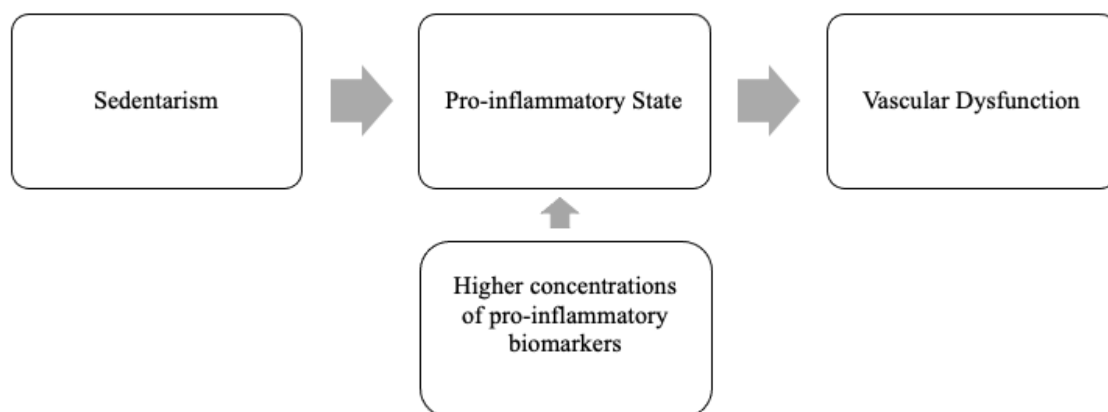
Experimental research provides insight into the mechanisms that may be involved in the association between sitting and vascular function. For example, Padilla et al., found that three hours in a seated position leads to acute vascular dysfunction as measured by leg blood flow shear stress in the femoral and popliteal arteries using flow-mediated dilation. The negative impact of prolonged sitting on vascular function appeared to have a ***sex-dependent*** effect such that the effect was less profound in young females [29]. Importantly, they found that resuming a walking activity for 10 minutes (~1000 steps) after sitting for six hours returns lower leg vascular function to baseline levels [29]. This effect of sitting and interrupted sitting on vascular function has been shown using measures of flow mediated dilation [30]. Unfortunately, studies of this nature have typically had small sample sizes (n = 8-20), do not adequately consider ***sex-differences***, and have a large range of prolonged sitting durations (1.5-6 hours) [31-34]. Thus, while experimental research has provided important insights, there are still several gaps that make it difficult to understand the relationship between sedentary time and vascular dysfunction.

Together, observational and experimental research suggest that prolonged sitting time can lead to vascular dysfunction, including impaired blood flow and endothelial dysfunction. Inflammatory factors may help to explain why prolonged sedentary time leads to vascular dysfunction. In homeostasis, endothelial function maintains vascular tone and blood fluidity, with minimal pro-inflammatory factors present. However, the initiation of an inflammatory response results in a loss of vasodilator and anti-thrombotic factors (e.g. Interleukin (IL)-13, Insulin-like Growth Factor (IGF-1), Angiogenin, and Vascular Endothelial Growth Factor (VEGF)), and an increase in vasoconstrictor, pro-thrombotic, and pro-inflammatory factors (e.g. Thrombopoietin, IL-8, Chemokine Ligand 5

(RANTES), IL-1β, and, Epidermal Growth Factor (EGF)), leading to poor blood flow and a higher risk of clotting [19]. Widlansky and colleagues also describe how increased adiposity, elevated C-reactive protein (CRP) levels, and chronic systemic infection are associated with vascular dysfunction. This can lead to a positive feedback loop, wherein higher levels of pro-inflammatory factors promote monocyte and T-cell adhesion, extracellular matrix digestion, and vascular smooth muscle migration and proliferation, thus resulting in further vascular dysfunction [19]. Inflammatory factors play a role in vascular function, blood flow, blood pressure, shear stress, and are fundamental to vascular health [23]. However, little is known of which inflammatory factors respond to acute bouts of sedentarism.

Presented in Figure 2.1 is a depiction of the concepts described above. Higher volumes of sedentary time induce a pro-inflammatory state, where concentrations of pro-inflammatory biomarkers and vascular factors may increase, thus leading to vascular dysfunction. This thesis aims to identify which vascular factors respond to prolonged periods of sitting, thereby uncovering the mechanistic link between sedentarism and vascular dysfunction.

Figure 2.1 Intersection of sedentarism, pro-inflammatory biomarkers and vascular factors, and vascular dysfunction



2.2.3. Metabolic Effects

Higher volumes of sedentary time have been linked with increased risk of obesity, weight gain, and type 2 diabetes [35-39]. Evidence suggests that higher volumes of sedentary time impact the metabolic profile [40]. Below is a review of observational and experimental studies investigating the link between sitting time and metabolic dysfunction.

Numerous ***population-based*** studies investigating the link between sitting and metabolic function have been published. In multiple cohort studies, there is an association between higher total sedentary time and/or prolonged periods of sedentary time and an increased risk of diabetes, hypertension, dyslipidemia, higher body mass index (BMI), metabolic syndrome criteria, and decreased physical activity [41, 42]. Importantly, in a population-based cohort study of adults aged 36-89 years (n = 739), waist circumference, BMI, high-density lipoprotein cholesterol, and triglycerides were significantly associated with device-measured sitting time and prolonged sitting time, with longer periods of accumulated sitting time having larger effects [43]. Clearly, there is an association between sedentary time and metabolic function, however, there is a lack of understanding of ***sex-specific*** differences and the underlying mechanisms; this has begun to be explored in experimental research.

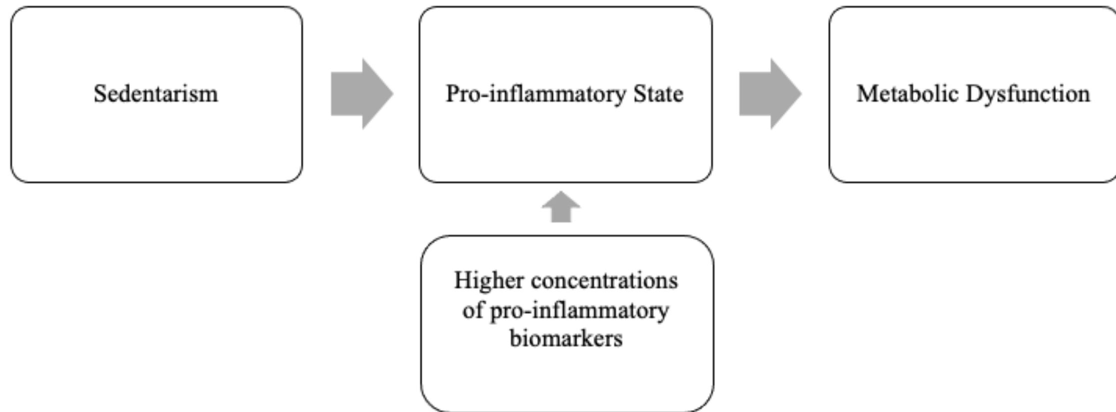
Experimental research provides further information into the mechanisms that may be involved in the association of sitting and metabolic dysfunction. In a review by Loh et al. of 42 studies using trials that either compared interrupted sitting, prolonged sitting, exercise prior to sitting, and exercise after sitting in adults with no major health conditions, they found that there is a significant reduction in glucose, insulin, and triacylglycerols with sedentary interruption protocols compared to prolonged sitting protocols. Among the 42 studies reviewed, sitting protocols ranged from 5-9 hours, interruptions occurred every 20-60 minutes, were generally of moderate intensity and ranged from 2-60 minutes in duration, with the most common being two minutes. Highlighted in the review is the need to understand how sex might mediate glucose responses, to develop ***sex specific*** physical activity break protocols, and an investigation into the underlying mechanisms for sex divergent metabolic responses [44]. Other studies support the findings of Loh et al., and describe an association between higher volumes of prolonged sitting and signs of low-

grade inflammation and oxidative stress, including decreased high-density lipoprotein cholesterol and increased low-density lipoprotein cholesterol, insulin sensitivity, and fasting triglycerides [45-48]. As sitting increases the metabolic profile becomes poorer; the reduction of lipoprotein lipase activity at the vascular endothelial level impairs the uptake of lipoprotein-derived fatty acids and may contribute to risks observed in metabolic diseases such as obesity, type 2 diabetes, and coronary heart disease [49]. One particularly strong study of adults aged 44-53 (n = 10 females and 6 males), examined interrupting sitting every 30 minutes from 8:00-18:00 with 3-minutes of low-to-moderate intensity walking for 4 weeks compared to a control group, living a habitual sedentary lifestyle. An oral glucose test, blood samples, and a muscle biopsy were taken, the experimental group increased walking time by 10.4 minutes/day, and subsequently mean fasting glucose levels, daily glucose variation, and low-density lipoprotein cholesterol levels were reduced [50]. While experimental research has provided more insights, there are still gaps in the specific mechanisms by which sitting and metabolic dysfunction are associated.

Taken together, observational and experimental research indicate an association between sedentary time and metabolic dysfunction. Inflammatory factors may have a role in metabolic dysfunction [51, 52], as chronic or excessive levels of inflammation have been linked with poor metabolic profiles and metabolic conditions (e.g. IL-8, IL-6, Tumour Necrosis Factor (TNF)-α, Adiponectin, Resistin, Fibrinogen, and C-Reactive Protein (CRP)) [51-58]. Evidence shows that inflammatory factors play a role in metabolic dysfunction, however, which inflammatory factors respond to acute bouts of sedentarism has yet to be fully determined.

Presented in Figure 2.2 is a summary of the concepts described above. High volumes of sedentarism result in a pro-inflammatory state, in which there are higher concentrations of pro-inflammatory biomarkers, thus resulting in metabolic dysfunction. This thesis aims to identify which biomarkers respond to prolonged bouts of sitting, to aid in determining the mechanistic link between sedentarism and metabolic dysfunction.

Figure 2.2 Intersection of sedentarism, pro-inflammatory biomarkers, and metabolic dysfunction



Summary: Accumulating high volumes of sedentary time is associated with poor vascular function and detrimental metabolic changes. Females have been observed to have higher total volumes of sedentary time, but shorter bout durations, and a less profound vascular dysfunction response to prolonged sitting.

Gaps: It is not clear why sedentary time leads to vascular and metabolic dysfunction. The pro-inflammatory state created during prolonged sitting could be implicated in this association, with concentrations of pro-inflammatory cytokines and vascular factors changing in response to prolonged sitting. However, it is not clear which factors are important to consider, or whether these associations differ by sex.

2.3. Biomarkers

In this section, biomarkers, that may explain the association between increased sedentary time and vascular dysfunction or metabolic disruption, are investigated further. This review is used to identify which biomarkers may be of particular interest when investigating prolonged sitting time and sedentary interruptions.

2.3.1. Cytokines, Chemokines, and Myokines

2.3.1.1. Cytokine, chemokine, and myokine structure and function

Cytokines are small proteins secreted by cells that impact cellular interactions [59]. They are categorized as either pro-inflammatory and anti-inflammatory in response to infection, immune response, inflammation, trauma, and/or stress, though some cytokines are pleiotropic, being both pro- and anti-inflammatory dependent on context [60, 61]. The function of pro-inflammatory cytokines is to help initiate the inflammatory cascade, whereas anti-inflammatory cytokines disrupt the inflammatory cascade, leading to the resolution of the inflammatory response. There is some nuance in cytokine classification because cytokine actions are dependent on stimulus, recruitment, and target which is known as a cytokine signature [61]. Cytokines also have a variable nomenclature and can be referred to as interleukins, chemokines, myokines, and/or growth factors [61]. **Chemokines**, also known as chemotactic or chemoattractant cytokines, are small proteins that signal through G protein-coupled heptahelical chemokine receptors on the cell surface. They are able to stimulate the migration of cells, particularly leukocytes, and play an important role in the development and homeostasis of the immune system. Chemokines have a similar structure to cytokines and typically have a name to reflect this, such as **IL-8** also being known as CXCL-8 [62]. **Myokines**, are cytokines and other peptides produced and released by muscle fibers. They mediate communication between muscles and other organs, such as the brain, bone, liver, pancreas, and vasculature, producing autocrine, paracrine, or endocrine effects [24]. For example, the myokine **IL-6** has an acute and chronic role in anti-inflammatory effects with exercise training [24].

Understanding the role and function of various cytokines can provide insight into sedentary physiology. Cytokines may be affected by sedentary time and play a role in the

inflammatory response associated with prolonged sitting [63]. Below is a review of literature that outlines the response of cytokines to sedentary time and exercise.

2.3.1.2. Cytokine response to sedentary time

Epidemiological studies suggest an association of sedentary time with pro-inflammatory and anti-inflammatory biomarkers [64]. For example, in a population-based cohort study of Irish males and females, aged 50-69 years old (n = 396), it was found that higher volumes of sedentary time were associated with a more pro-inflammatory profile, with higher concentrations of Complement Component 3 (C3), C-reactive Protein (CRP), Interleukin (IL)-6, Tumour Necrosis Factor (TNF)-α, Leptin, and White Blood Cells (WBC); there were lower concentrations of pro-inflammatory cytokines observed with light and moderate-to-vigorous intensity physical activity (MVPA) [64]. Similar findings were observed in a cross-sectional study using the National Health and Nutrition Examination Survey data from 2003/2004 and 2005/2006 and in a sample of adolescents aged 13.0-16.99 years (n = 281 male and 337 female), from the Health Lifestyle in Europe by Nutrition in Adolescence (HELENA) study [65, 66]. In two other studies investigating IL-6, IL-10, TNF-α, and CRP in blood samples using accelerometer data, one investigating cytokines among participants admitted to hospital with acute ischemic stroke (n = 257) and the other investigating cytokines in obese and overweight pregnant females (n = 46), it was found that pro-inflammatory cytokines were associated with higher volumes of sedentary time [67, 68]. An observational study investigating ***sex-differences*** in cytokines by Bergens et al. found that in females a higher amount of total sedentary time was related to higher levels of pro-inflammatory biomarkers (IL-6 and Fibrinogen), whereas among males higher amounts of total sedentary time were related to lower levels of the anti-inflammatory biomarker, IL-10 [69].

Clearly there is a link between sedentarism and some commonly investigated pro-inflammatory biomarkers such as CRP and Fibrinogen and cytokines such as IL-6 and TNF-α. However, the full scope of which cytokines respond to acute bouts of prolonged sitting has yet to be explored, with little research investigating ***sex-specific differences*** in cytokine response.

2.3.1.3. Cytokine response to exercise

Experimental research has begun to investigate the associations between sedentary time, physical activity, and cytokine responses. In a randomized control trial of participants with a type 2 diabetes diagnosis within the past 6-months (n = 184 males and 101 females), participants provided a fasted venous blood sample and wore an accelerometer for seven days. Falconer et al. found that there were no sex-related differences in total sedentary time, but there was an association between sedentary time and IL-6 among males and females [70]. However, in a review by Gillum et al., it is stated that females appeared to have a more active immune system compared to males of a matched age and cytokine response appeared to be menstrual phase dependent [71]. In a study investigating the response of biomarkers to exercise, among young sedentary (n = 15), young active (n = 16), old sedentary (n = 23), and old active (n = 14) males and females, after acute exercise a reduction in Interferon Gamma-induced Protein (IP)-10, IL-6, and RANTES was observed in all groups [72]. There also appeared to be a protective influence of long-term physical activity against the effects of sedentary aging, particularly that older, sedentary individuals are less able to induce favourable cytokine changes with exercise. Additionally, similar to Gillum et al., the study states that research in females must take into consideration changes in sex hormone concentrations during the menstrual cycle, as estrogen can modulate the immune response [72]. One study investigated a sample of sedentary females (n = 15; age 20-24) in the follicular phase of the menstrual cycle. After a single bout of exercise (1 hour of cycling at 70% VO_2max) there was a decrease in serum concentration of RANTES from baseline [73]. Exercise intensity may also influence cytokine response; for example, a randomized control trial investigated the IL-8 response to different exercise intensities. Participants were randomly allocated to six weeks of HIIT (n = 12) or MICT (n = 11). Both exercise intensities lead to improvements in VO_2max and a reduction in IL-8 concentrations, however, MICT lead to a greater decrease in IL-8 concentrations compared to HIIT [74]. Thus, there appears to be an interaction of age, sex, physical activity levels, and intensity on the association of sedentary time and cytokines.

Previous work from our lab has focused on the response of salivary cytokines to prolonged and interrupted sitting. A sample of healthy males and females (n = 10) completed a prolonged four-hour sitting session and an interrupted 4-hour sitting session,

with a three-minute cycling protocol every hour. Saliva was collected to measure cytokines using an enzyme-linked immunosorbent assay (ELISA) and lower leg swell was measured using a strain gauge. Saliva sample levels of TNF-α and IL-6 were below the limit of quantification, and IL-1β was not observed to be significantly different between the prolonged versus disrupted sitting protocols. However, IL-8 concentrations increased during the prolonged sitting and decreased during the disrupted sitting sessions. In other words, levels of this pro-inflammatory marker increased while sitting and decreased when regular movement was incorporated into the sitting session. Leg swell was increased in the prolonged sitting session and attenuated during the interrupted session. Some limitations of this study include lack of sex-based analysis, using a cycle ergometer for high intensity exercise, and not performing a randomized cross-over. In other work from our lab, a sample of young, healthy males and females (n = 24, 50% female) participated in a study of the same protocol as this thesis, but both saliva and blood were collected and ELISAs were used to investigate the concentration of IL-8 in saliva and capillary plasma. Concentrations of IL-8 in saliva increased in both the prolonged and interrupted sitting protocols, with a more significant increase in the prolonged protocol. There was no increase in IL-8 concentration in the sub-sample of males in the interrupted protocol, and there were no significant findings for IL-8 in the plasma. This research concludes that salivary concentrations of IL-8 appear to increase, that interrupting this sitting with short bouts of walking seems to blunt this response, and that sex seems to modulate this response. A limitation of this study is the high individual variability among participants and ELISAs [75].

Thus, work is needed to understand whether lighter intensity interruptions, which may be more feasible for the general population, would lead to better outcomes, and whether there are differences in the responses of males and females. Summarized in Table 2.1 is a review of the literature of cytokine responses to sedentary time and exercise. Of note are the cytokines IL-8, IL-3, IL-6, and RANTES, which may warrant further investigation. Additionally, the investigation of which other cytokines, not commonly mentioned in the literature, respond to acute bouts of sitting may be needed to link sedentarism to vascular and metabolic dysfunction.

Table 2.1 Current research on cytokine responses to sedentary behaviour and exercise

Study	Study Design	Cytokine	Classification	Effect
Sedentary behavior in obese pregnant women is associated with inflammatory markers and lipid profile but not with glucose metabolism [68]	Longitudinal observational	IL-6	Myokine	Females spent up to 60% of their time sedentary during pregnancy with a mean of 52.5 ± 11.1 hours/week. Sedentary time was associated with lower IL-6 at 24 weeks. Sedentary time was associated with higher IL-10 at 24 weeks. Sedentary time was associated with higher TNF- α at 24 weeks.
		IL-10	Myokine	
		TNF- α	Myokine	
Diet as a moderator in the association of sedentary behaviors with inflammatory biomarkers among adolescents in the HELENA study [65]	Cross-sectional	IL-10	Myokine	Sedentary time of 226.60 ± 151.39 minutes/day for males and 157.98 ± 111.76 minutes/day for females. Sedentary time was associated with higher IL-10. Sedentary time was associated with higher TNF- α. A healthy diet attenuates the effect of sedentary behaviours on the pro/anti-
		TNF- α	Myokine	
		IL-6	Myokine	
		IL-10	Myokine	
		IL-6	Myokine	

				inflammatory cytokine ratio.
Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: a multicentre prospective ischemic stroke cohort study [67]	Prospective cohort	IL-6	Myokine	Mean sedentary time was calculated as 9.7 ± 1.9 hours/day. Sedentary time was significantly associated with levels of hsCRP and IL-6 when adjusting for age, sex, waist circumference, and creatinine. No association between sedentary behaviour and IL-10.
		High Sensitive C-reactive Protein (hsCRP)	Acute Phase Protein	
		IL-10	Myokine	
Disrupting prolonged sitting reduces IL-8 and lower leg swell in active young adults [63]	Experimental	IL-8	Chemokine	Concentrations of salivary IL-8 increase during prolonged sitting and decrease during interrupted sitting.
Does replacing sedentary behaviour with light or moderate to vigorous physical activity modulate inflammatory status in adults? [64]	Population based cohort	C-Reactive Protein (CRP)	Acute Phase Protein	Higher volumes of sedentary time were associated with higher concentrations of CRP, IL-6, TNF-α, and White Blood Cells. There were lower concentrations of pro-inflammatory cytokines observed with light, and moderate-to-
		IL-6	Myokine	
		TNF-α	Myokine	
		White Blood Cells	N/A	

				vigorous physical activity.
Sedentary time and cardio-metabolic biomarkers in US adults: NHANES 2003–06 [66]	Cross-sectional	CRP	Acute Phase Protein	Linear association between total sedentary time and CRP, with females having higher CRP than males.
Sedentary patterns and systemic inflammation: sex-specific links in older adults [69]	Observational	CRP	Acute Phase Protein	No differences in CRP concentration between males and females. Females with a higher amount of total sedentary time were associated with higher levels of IL-6, whereas males with a higher amount of total sedentary time were associated with lower levels of IL-10.
		IL-6	Myokine	
		IL-10	Myokine	
A review of sex differences in immune function after aerobic exercise [71]	Narrative review	IL-8	Chemokine	Females of a reproductive age have more active immune systems compared to males of a matched age, indicating that cytokine response could be menstrual phase dependent, however there was no difference found between serum IL-8

				immediately after and 1.5 hours after completing a marathon.
Endothelial dysfunction and specific inflammation in obesity hypoventilation syndrome [76]	Randomized control trial	RANTES	Chemokine	RANTES is significantly increased in Obesity Hypoventilation Syndrome patients, compared to obese patients with normal concentrations of CO_2 in the blood.
Acute, Exercise-Induced Alterations in Cytokines and Chemokines in the Blood Distinguish Physically Active and Sedentary Aging [72]	Cross-sectional	IL-3	Cytokine	Participants completed an indirect aerobic capacity cycle test then a 30-minute cycle session at 50% of the maximum attained VO2peak. Young, active individuals have a significantly higher fraction of detectable sample cytokines. IL-8, Growth Regulated Protein, IL-18, Interferon Gamma-induced Protein (IP)-10, and Chemokine Ligand 5 (RANTES), were higher and IL-12 was lower
		Plasma fractalkine	Cytokine	
		TNF-α	Myokine	
		IL-8	Chemokine	
		IL-18	Myokine	
		Interferon Gamma-induced Protein (IP)-10	Chemokine	
		RANTES	Chemokine	
		IL-12	Cytokine	

				in the older age group regardless of physical activity level. Concentrations of IL-3, Plasma fractalkine, and TNF-α are higher in older sedentary individuals versus younger sedentary individuals but lower in older physically active participants compared to older sedentary participants. After acute exercise a reduction in IP-10, IL-6, and RANTES was observed in all groups. Physical activity had no impact on IL-8 or IL-12.
Sedentary time and markers of inflammation in people with newly diagnosed type 2 diabetes [70]	Randomized control trial	IL-6	Myokine	For every additional hour spent sedentary, IL-6 was observed to be 8% lower in males, and 12% lower in females. At the 6-month follow up, a reduction in sedentary time observed in females was associated with a
		CRP	Acute Phase Protein	

				42% reduction in CRP.
Salivary concentrations of IL-8 and IL-1ra after HIIT and MICT in young, healthy adults: A randomized exercise study [74]	Randomised control trial	IL-8	Chemokine	Both HIIT and MICT lead to improvements in VO_2max and a reduction in IL-8 concentrations. However, MICT leads to a greater decrease in IL-8 concentrations compared to HIIT.
A single session of intense exercise improves the inflammatory response in healthy sedentary women [73]	Experimental	RANTES	Chemokine	After a single bout of exercise in a sample of sedentary females, in the follicular phase of the menstrual cycle, the serum concentration of RANTES was shown to decrease from baseline in response to exercise. GRO and MCP-1 increased post exercise.
		GRO	Chemokine	
		MCP-1	Chemokine	
Shorts bouts of walking attenuates the response of IL-8 to prolonged sitting in healthy adults [75]	Randomized cross-over trial	IL-8	Chemokine	Concentrations of IL-8 in saliva increased in both the prolonged and interrupted sitting protocols, with a more significant increase in the prolonged protocol.
Effects of acute physical exercise on	Experimental	Monocyte Chemoattractant	Chemokine	Of a sample of 30 sedentary males, divided

oxidative stress and inflammatory status in young, sedentary obese subjects [77]		Protein (MCP)-1		into normal weight, overweight, and severe obesity categories. Acute exercise produced a significant decrease in MCP-1 in the normal weight group.

2.3.2. Growth, Endothelial, and Vascular Factors

2.3.2.1. Growth, endothelial, and vascular factor structure and function

Growth factors are a sub-group of cytokines, which are molecules that can impact the growth of cells. Growth factors can promote or inhibit mitosis and affect cellular differentiation. Some cytokines which affect cell growth and differentiation are also growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). Whereas vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are growth factors specific to particular cell types [78]. EGF is a factor that stimulates the proliferation of various cells, particularly fibroblasts and epithelial cells. Its primary roles it to activate EGF receptor (EGFR), which in turn initiates intracellular signalling, leading to the differentiation, maturation, and survival of a variety of neurons [79]. VEGF is a factor that regulates blood and lymphatic vessel development and homeostasis. VEGF is predominately produced by endothelial, hematopoietic, and stromal cells in response to hypoxia and by the stimulation of other factors such as Transforming Growth Factor Beta (TGF-β), interleukins, or PDGF [80]. **Endothelial cells and factors** control vascular homeostasis and have important autocrine, paracrine, and endocrine actions. Endothelial cells help to regulate blood flow and blood pressure by releasing vasodilator and vasoconstrictor substances. The endothelium is also involved in platelet activation, aggregation, inflammation, immune modulation, vascular smooth muscle cell proliferation, and angiogenesis [81]. **Angiogenin** induces angiogenesis by activating endothelial and smooth muscle cells, leading to cell migration and proliferation

[82]. Below is a review of the current literature of the response of growth, endothelial, and vascular factors to sedentary time and exercise.

2.3.2.2. Growth, endothelial, and vascular factor response to sedentary time

Sedentary behaviour and bed rest studies have begun to investigate the associations between sedentary time and growth, endothelial, and vascular factor response. In a seven-day bed rest study of healthy young males (n = 10; age = 23 ± 1 years), bed rest did not lead to significant changes in VEGF protein expression(−13 ± 10%; P > 0.05) [83]. In another seven-day bed rest study of young, healthy males (n = 12), the lack of VEGF response was replicated, however, acute exercise increased VEGF mRNA content in skeletal muscle before bed rest, with responses blunted after bed rest [84], indicating that prolonged sedentary time may influence VEGF response to exercise. Alternatively in a study investigating serum levels of endostatin, VEGF, IL-8, angiogenin, and basic fibroblast growth factor (bFGF) (n = 82 hypertensive participants; n = 34 healthy participants), healthy individuals were characterised by decreased concentrations of endostatin, VEGF, CRP, and IL-8, and increased concentrations of angiogenin and bFGF [85]. VEGF may also be **sex-dependent** as a cross-sectional study using the ActiveBrains project, overweight/obese children aged 8-11 (n = 97, 58% male) found VEGF concentrations were greater in males (37.7 (34.8) pg/mL) than in females (34.5 (51.1) pg/mL) though this difference was not significant [86]. In a cross-sectional study from the Korea National Health and Nutrition Examination Survey (n = 33, 403), they investigated possible mechanisms to explain the association between sedentary behaviour, physical activity, and colorectal cancer. The study states that prolonged sedentary behaviour and low physical activity can lead to metabolic dysfunction, such as insulin resistance, leading to hyperinsulinemia and hyperglycemia. These conditions have been shown to activate Insulin-like Growth Factor (IGF)-1, and upregulate glucose transporter, EGF expression and subsequently activate EGFR, respectively [87, 88].

Clearly there is a link between sedentary time and growth, endothelial, and vascular factors, however, most research investigates common factors such as VEGF and EGF with little research investigating alternate factors and sex-specific differences in factor response.

2.3.2.3. Growth, endothelial, and vascular factor response to exercise

Exercise studies have begun to investigate the mechanistic links between growth, endothelial, and vascular factor responses and exercise. In a study of male endurance athletes (n = 8) and sedentary males (n = 8), who completed an acute exercise protocol at 50% maximum power output for one-hour, peak post-exercise VEGF increased independent of training status [89]. In two other studies of young, sedentary individuals (n = 6 males and 1 female) and healthy males (n = 8), participants completed an acute resistance training intervention or 7 sessions of 45 minute, 60 rpm knee extensions via ergometer, respectively, plasma VEGF levels and VEGF mRNA levels were also increased post exercise [90, 91]. Gómez-Pinilla and Feng, state that exercise enhances the proliferation of endothelial cells throughout the brain, hippocampal IGF gene expression, and serum levels of IGF and VEGF, supporting exercise induced neurogenesis and angiogenesis [92]. Similarly, in a study of 50 healthy males (n = 34) and females (n = 16) divided into three ethnic groups, European (n = 25), Middle Eastern (n = 10), and South Asian (n = 15) VEGF plasma concentrations were influenced by all exercise intensities and there were no significant differences observed based on sex or ethnic group. Baseline VEGF concentrations were higher in females, yet showed a similar response to exercise as males [93]. In a study investigating EGF in older males (68 ± 1 years) (n = 9) and younger males (28 ± 2 years) (n = 11) it was found that after 8-weeks of 3 sets of 20-second static sprints two times per week, EGF was decreased from 142 (20) pg mL^{-1} at baseline to 100 (12) pg mL^{-1} after exercise intervention in the older males, similar to the baseline of the younger males (60 (12) pg mL^{-1}) who did not undergo training [94]. Similar findings were observed in a study of sedentary males (n = 30, aged 20-45), divided into three groups (normal weight: BMI < 25 kg/m^2; overweight to moderate obesity: 25–35 kg/m^2; severe obesity: 35–40 kg/m^2). Blood samples collected prior to and post a 20-minute run at ~70% VO_2max, showed a significant decrease in MCP-1 in the normal weight group post exercise, and a decrease in EGF levels in all groups post exercise [77]. A study of 68 healthy adults (n = 33 males and 35 females, aged 21-88 years), using tear samples, showed that EGF concentrations were significantly higher for males (3.4±0.3 ng/mL) than for females (2.4±0.3 ng/mL; p = 0.043) [95]. However, a study of Type I or Type II diabetic individuals (n = 21) matched with healthy individuals (n = 21), observed that there were

no statistically significant differences in salivary protein concentrations between males and females in the diabetic or control groups [96]. In a study comparing young, male athletes (n = 14, 5 athletes tested twice) and healthy, active adults (n = 9 males and 11 females, age 68-88 years), serum angiogenin increased after intense exercise and showed no difference between males and females [97]. In a review of the literature, it is stated that exercise induces angiogenesis, increases angiogenin, and that both resistance and aerobic exercise have positive effects, but aerobic exercise seems to have a more pronounced effect [98].

Thus, there appears to be an influence of age, sex, and physical activity levels on the association of sedentary time and growth, vascular, and endothelial factors. Summarized in Table 2.2 is a review of the literature about the response of growth, endothelial, and vascular factors to sedentary time and exercise. VEGF, EGF, and Angiogenin are factors that warrant further investigation into their response to prolonged sitting and sedentary interruptions. Additionally, further investigation in which other factors may respond to acute bouts of sitting and if there are sex-dependent differences in factor response may help to better understand the mechanistic link between sedentarism and vascular and metabolic dysfunction.

Table 2.2 Current research on growth, endothelial, and vascular factor responses to sedentary behaviour and exercise

Study	Study Design	Growth Factor	Sample Source	Effect
Does replacing sedentary behaviour with light or moderate to vigorous physical activity modulate inflammatory status in adults? [64]	Population based cohort	Leptin	Serum and Plasma	Higher volumes of sedentary time were associated with higher concentrations of Leptin.
Acute, Exercise-Induced Alterations in Cytokines and Chemokines in the Blood Distinguish Physically Active and Sedentary Aging [72]	Cross-sectional	Growth Regulated Protein Platelet Derived Growth Factor (PDGF)-AA Epidermal Growth Factor (EGF) Vascular Endothelial Growth Factor (VEGF) TGF-α	Plasma	Participants completed an indirect aerobic capacity cycle test then a 30-minute cycle session at 50% of the maximum attained VO_{2peak}. Young, active individuals have a significantly higher fraction of detectable sample cytokines. Growth Regulated Protein, Platelet Derived Growth Factor (PDGF)-AA, and PDGF-BB were higher and Vascular Endothelial Growth Factor (VEGF), and Epidermal Growth Factor (EGF) were lower in the older age group regardless of physical activity level. TGF-α levels were higher among the old sedentary group versus the young sedentary group.
Bed rest reduces metabolic protein content and abolishes exercise-induced	Experimental	VEGF mRNA	Skeletal Muscle	VEGF concentrations did not change significantly with 7-days of bed rest in young, healthy males. After

mRNA responses in human skeletal muscle [84]				acute exercise prior to bed rest VEGF mRNA content in skeletal muscles increased, however this response to acute exercise was not observed post bed rest.
Effects of acute physical exercise on oxidative stress and inflammatory status in young, sedentary obese subjects [77]	Experimental	EGF	Blood	Of a sample of 30 sedentary males, divided into normal weight, overweight, and severe obesity categories, EGF concentrations were higher in the normal weight individuals pre and post exercise. Acute exercise produced a significant decrease in EGF concentrations in all BMI categories.
Circulating plasma VEGF response to exercise in sedentary and endurance-trained men [89]	Experimental	VEGF and Soluble VEGF Receptor (sFlt-1)	Plasma and Serum	In male endurance athletes and sedentary males who completed acute exercise at 50% maximum power output for one-hour, peak post-exercise VEGF increased independent of training status.
Acute resistance exercise increases skeletal muscle angiogenic growth factor expression [90]	Experimental	VEGF, VEGF mRNA, VEGF Receptors	Muscle and Plasma	In young, sedentary individuals after an acute resistance training protocol, skeletal muscle VEGF mRNA and plasma VEGF protein was increased at 2- and 4-hours post-exercise.
Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged	Experimental	VEGF and VEGF mRNA	Muscle and Blood	In healthy males, who participated in a 10-day, 7-session exercise intervention, there was an exercise-induced increase in VEGF mRNA and protein levels.

exercise training [91]				
Effects of different intensities of exercise on concentrations of Endostatin and VEGF in the plasma of healthy volunteers [93]	Experimental	VEGF	Plasma	In a study of healthy individuals (n = 22; 16 male and 6 female), VEGF concentration was influenced by all exercise intensities.
Sedentarism, physical activity, steps, and neurotrophic factors in obese children [86]	Cross-sectional	VEGF	Plasma	In a study of obese/overweight children, aged 8-11 years (n = 88), VEGF concentrations were greater in males (37.7 (34.8) pg/mL) than in females (34.5 (51.1) pg/mL) though not significant.
Sprint interval training (SIT) reduces serum epidermal growth factor (EGF), but not other inflammatory cytokines in trained older men [94]	Experimental	EGF	Serum	In a study of older males (n = 9) and younger males (n = 11), EGF levels decreased from 140 (20) pg/mL at baseline to 100 (12) pg/mL after eight-weeks of exercise training in the older males. The post-exercise intervention EGF levels of the older males were then similar to the baseline EGF levels of the young males (60 (12) pg/mL).
The effects of age, gender, and fluid dynamics on the concentration of tear film epidermal growth factor [95]	Observational	EGF	Tear Samples	In a sample of healthy adults (n = 33 males and 35 females), EGF concentrations were determined using ELISA. EGF tear concentrations were significantly higher for males (3.4±0.3 ng/ml)

				than for females (2.4±0.3 ng/ml).
Salivary EGF levels reduced in diabetic patients [96]	Observational	EGF	Saliva	In a sample of individuals with Type I or Type II diabetes, matched with a sample of healthy individuals (n = 42), salivary EGF concentrations were significantly lower for diabetic patients compared to the control patients. There were also no significant differences in salivary protein concentrations between males and females in the diabetic or control groups.

Summary: Observational and experimental research indicate that there is an association between sedentary time and concentrations of pro and anti-inflammatory biomarkers in the serum and saliva. Higher volumes of sedentary time are associated with higher levels of pro-inflammatory biomarkers, and vascular and endothelial factors. Outlined in Table 2.1 and Table 2.2 is a summary of the current studies investigating biomarkers and their response to sedentary time and exercise.

Gaps: There is a lack of research available on young and healthy adults, and few studies have assessed how sex alters the response of various biomarkers to sitting. Importantly, the bulk of the literature has focused on a handful of well-known biomarkers, while many others have been ignored.

2.4. References

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3. Methodological Approaches

In this section I will review the literature on the use of saliva as a method for assessment of biomarkers and introduce different methods of analyzing saliva.

3.1. Salivary Biomarkers

Biomarkers can be measured using different bodily fluids, such as blood, serum, saliva, plasma, urine, and muscle biopsies. There are pros and cons associated with each of these methods. Previously, blood and muscle biopsies were considered the "gold standard" in the analysis of biomarkers in exercise physiology [1]. However, these techniques are invasive. Saliva sampling is a non-invasive method of measuring changes in biomarker concentrations. While serum and plasma have established efficacy and accuracy [2, 3], a consequence of drawing blood is that acute inflammation can occur due to the stress induced within the participant during blood collection. Alternatively, saliva sampling is a safe and non-invasive method, that does not pose the same risk of inducing inflammation during collection [2, 3].

Biomarker responses have been correlated when measured using venous or capillary blood sampling [4, 5]. Saliva, plasma, and serum samples, however, have not been shown to be correlated, though saliva still remains a valid measure [6]. In a study completed by Kaufman and Lamster on the diagnostic applications of saliva and a study completed by Miller et al., on the developments in salivary diagnostics, saliva has shown to be an accurate method of sampling and to be applicable for multiple clinical settings [7, 8]. The differing results from saliva, venous, and capillary samples does not indicate that salivary samples are inaccurate, rather that they simply provide different results when compared with serum and plasma [6].

Saliva sampling is a feasible and practical option to measure biomarker response to stressors. In a review completed by Lindsay and Costello, it was found that non-invasive methods allow for (1) rapid sample collection, (2) personalized timing of sample collection, (3) reduced risk of cross-contamination, (4) limited increases in stress hormone concentrations, (5) simplicity of collection devices, and (6) reduced distress/pain to the participant [1]. Lindsay and Costello also state that with the improvement of diagnostic

methodologies, such as immunoassays, chromatographic assays, and spectrometric assays, that saliva can provide an adequate molecular profile and that it enables suitable analysis [1]. In a study completed by Rutherfurd-Markwick et al. they describe saliva as a "relevant fluid for clinical and forensic diagnosis, as well as for analysis in sport, exercise and nutrition research" [9]. They state that saliva allows for easy, non-invasive collection, that saliva is more stable than blood, therefore requires less preparation for analysis, and provides a large number of analytes [9]. A review by Szabo et al. found that 17 cytokines, salivary markers of inflammation, responded to an acute stressor and were reported as detectable in saliva across multiple studies [3]. Overall, saliva collection and analysis is well suited for the proposed research.

3.2. Saliva Sample Collection

There are some confounders that require consideration when using saliva. These are briefly outlined below.

Time of day is an important consideration for saliva sampling. Previous research has shown that cytokines measured in blood can follow a circadian rhythm, with concentrations being higher at certain points of the day [10]. It is currently unknown if salivary cytokines follow a similar rhythm; however, to account for this potential confounder it is important to collect samples at the same time of day, as is currently done for blood samples.

Baseline salivary sampling is especially important when investigating salivary biomarkers in response to a stimulus. Using a baseline measurement would allow for validation of experimental paradigms and investigation of the magnitude of change [10]. It is especially important to have baseline measures when completing a control group or cross-over design to ensure that differences were truly due to the response to the stressor rather than individual differences [10].

There are also participant specific considerations to be managed. These include oral health, overall health, caffeine use, alcohol use, drug use, smoking, exercise, medication use, diet (food/drink), fever, and acute infection [10]. These characteristics present potential sample confounders and must be determined to elucidate potential biases during sample collection. Factors to be considered for inclusion and exclusion criteria, need to be accounted for during the experimental protocol. The following is an outline of the

necessary considerations for sample collection. Oral health must be assessed prior to participation, as poorer oral health or recent dental procedures could increase local inflammation. An individual with good oral health would theoretically have salivary biomarkers more representative of systemic inflammation [10]. Previous research has indicated that participants should be controlled for age, sex, and body mass index and excluded based on acute exercise, sleep deprivation, medication use, alcohol dependence, and smoking [11]. Individuals with autoimmune diseases or diseases that affect saliva production should also be excluded as they could have atypical immune responses and/or saliva production [10]. Research has shown that smoking can impact cytokine concentrations, as nicotine triggers neutrophil activation. This can lead to cytokine production, oxidative stress, increased risk of poor oral health, and chronic disease which are associated with increased levels of systemic inflammation [12-14]. It is recommended to limit food intake prior to collection to reduce debris and the risk of dilution. Water is recommended to promote the production of saliva. It is recommended to avoid caffeine and alcohol consumption use at least 3 hours prior to collection, as it can impact the accuracy of results. Exercise has also been shown to increase inflammation and so it is recommended that participants avoid exercise for at least 1 hour prior to study participation [10].

Saliva collection can vary in methodology including passive, drool, draining, swab, spitting, and suction methods [15]. It is recommended that sampling remains consistent through a study's protocol [15]. The collection of Whole Mouth Fluid (WMF), also known as Whole Mouth Saliva (WMS), is used interchangeably with the term "saliva" and is comprised of saliva, gingival crevicular fluids (GCF), oral mucosal transudate, mucus from the nasal cavity and the pharynx, as well as products resulting from bacterial metabolism, food debris, and desquamated epithelial cells [16]. There are some benefits to using various methods of collection, particularly, using a Salivette, which is a polypropylene tube with a perforated inset tube, and an absorbent wad made from cotton, polyester, or polyethylene. It is placed in the mouth, against the cheek or under the tongue to absorb WMF [17]. Studies have used a variety of time in the mouth to collect ranging from 2-10 minutes [17, 18]. This method of collection reduces the risk of cross-contamination and biohazards within the lab. It also is non-invasive and provides an adequate volume within a short time period. The absorbent material can act as a filter to remove large debris and avoid future assay

interference [16]. Studies have shown that a minimum of 0.5 mL of saliva is required during collection, with most devices resulting in 1.0-1.2 mL collected [16]. A study completed by Gröschl et al., stated that participants found the Salivette with cotton the most practical and convenient method. They also found that the Salivette produced the most sample (2 mL) [17]. Collection using the Salivette method is therefore feasible, as it is convenient for the participant, non-invasive, and provides adequate sample quantities.

Saliva sample processing and storage is simple in comparison to other bodily fluids, such as blood, serum, and urine. Immediate processing and storage are recommended. Centrifuging at 2, 600g for 15 minutes is recommended by Henson and colleagues, as well as storage at -80°C [19]. Salimetrics SalivaBio, Salimetrics LLC, State College, PA, USA, recommends that samples are centrifuged for 15 minutes at 1,500g, then stored at -20°C for periods up to four months and -80°C for periods longer than four months. In a study by Garde and Hansen, centrifuged saliva samples were stored at -80°C for up to 1 year successfully [20]. The Salivette® from Sarstedt yields adequate sample within 2 minutes when centrifuged at 1,000g. In a study by Mohamed et al., saliva samples were thawed then centrifuged at 10,000g for 10 minutes [21].

Table 3.1 Benefits and limitations of saliva sampling

Benefits	Limitations
– Safe – Non-invasive – Rapid sample collection – Low cost – More stable than blood – Easier storage – Less preparation for analysis – Personalized sample timing – Reduced risk of cross-contamination – Less distress and pain for the participant – Simplicity of collection – Reduced risk of inducing inflammation – Limited increase in stress hormone concentration – Adequate number of analytes – Less biosafety risk (lower biosafety level classification) – Requires less specialized training to collect – Collection provides adequate volume of sample	– Not the "gold-standard" – Might offer less specific analysis – Potentially lower concentrations of analytes – Potential confounders due to oral health, diet, etc.

Saliva is a biological fluid that can be used diagnostically for the analysis of biomarkers. In the previous discussion and summarized in Table 3.1, there is sufficient evidence to support the use of saliva as a method of collection for the analysis of cytokines. Saliva provides a feasible, time conscious, and consistent volume, method of collection.

3.3. Sample Analysis

Individual cytokines can be assessed using Enzyme-Linked Immunosorbent Assays (ELISA), but in order to detect the responsiveness of multiple cytokines, we can use antibody array kits, potentially followed by ELISA. Microarray kits are probe-based kits used to detect the presence of a range of specific molecules and can be designed to detect DNA, RNA, or specific proteins, including cytokines and growth factors. DNA and RNA-based microarrays use nucleic acid to capture probes. In contrast, most cytokine detection

membrane arrays use antibodies for target detection. ELISAs are a plate-based technique, that can detect and quantify soluble substances such as cytokines and antibodies. Both cytokine arrays and ELISAs use a specific antibody-antigen interaction. However, membrane array approaches allow for simultaneous detection of several biomarkers at once and are semi-qualitative, while ELISAs quantify precise biomarkers. A cytokine array is analyzed using the same method as a chemiluminescent western blot. See Table 3.3, for an overview of current research completed using microarrays, saliva, and humans. See Table 3.4 for an overview of microarray kits.

A Bradford Protein Assay is used to detect the concentration of total protein in a sample. The binding of protein molecules to Coomassie Dye results in a colour change [22]. The Bradford analysis is a rapid and accurate method to estimate protein concentration, and is a simpler, faster, and more sensitive method compared to other techniques [23]. A Bradford analysis can be used to determine the concentration of total protein within a saliva sample, to then determine if dilution is required depending on the sensitivity of the subsequent analysis. The Bradford Protein Assay protocol used in this study required 10 µl for analysis and provides results in µg/mL [24, 25].

Immunoassays are used to quantify molecules of interest using antibody reagents. Immunoassays are developed by establishing critical success factors and using a precision profile to determine a quantifiable range. It requires various parameters including analyte to be measured, sample matrices (serum, plasma, saliva, etc.), source of antibody, analyte standards, and detection reagents, a detection mode (colorimetric, fluorescence, chemiluminescence), type of immunoassay (sandwich, competitive, or antigen-down), and expected analyte concentration ranges [26, 27]. A Sandwich Immunoassay uses a matched pair of antibodies, one antibody is used to stick the analyte to the solid surface, the other is used to detect and bind to the analyte, and the antigen then becomes 'sandwiched' between the two antibodies [26]. A Competitive Immunoassay uses a specific antibody for the specific analyte, often used for small analytes [26]. An Antigen-Down Immunoassay binds the antigen to the solid surface by coating the surface in the antigen, the signal is proportional to the amount of antibody present [26].

The reading of an immunoassay can be completed using Spectrophotometric/Colorimetric, Fluorescence, or Chemiluminescence Plate readers. Spectrophotometric and Colorimetric plates use lamp sources and Photomultiplier Tubes (PMT). Readers have a linear range from about 0-4 Absorbance Units (AU) [26]. Fluorescence plate readers use excitation and emission filter sets as well as lamp sources and PMTs, usually provided in Relative Fluorescence Units (RFU) [26]. Chemiluminescence readers have photomultipliers to detect light from the chemical reaction, no lamp sources are required and they usually have a larger range for greater sensitivity, measured in Relative Light Units (RLU) [26]. Most analyses use approximately 100μL for analysis.

There has been some research investigating the use of microarrays in exercise physiology studies, this can be used to inform our research on sedentary physiology. Studies using microarrays and exercise physiology focus on the analysis of DNA, RNA, or protein and can provide insight into the underlying mechanism of exercise-induced stress, training adaptions, and immune function [28]. Table 3.2 is a summary of research completed investigating exercise physiology and the use of microarray kits.

Table 3.2 Studies using microarray methods to investigate effects of exercise

Study	Microarray Kit Used	Investigated	Sample Population	Sample Size (n)	Study Interest
Aerobic exercise augments muscle transcriptome profile of resistance exercise [29]	Affymetrix HuGene-2.1-st platform	DNA from muscle biopsy of the vastus lateralis muscle	Moderately trained males (age 26 ± 5 years)	10	Effect of acute aerobic exercise on transcriptional response to subsequent exercise
Angiopoietin-like 4 Enhances the Proliferation and Migration of Tendon Fibroblasts [30]	Agilent SurePrint G3 Human GE 8x60K Microarrays (AMDID 028004; Agilent, Santa Clara, CA).	mRNA from tendon fibroblasts	Healthy individuals	3	Explore mechanisms of Angiopoietin-like 4 (ANGPLT4) on tendon cells
Expression of genes involved in fatty acid transport and insulin signaling is altered by physical inactivity and exercise training in human skeletal muscle [31]	Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix)	RNA from muscle biopsy of the vastus lateralis muscle	Healthy males (6) and individuals with spinal cord injuries (8)	14 (6 deconditioning and 8 electrical stimulation exercise training)	Muscular gene expression levels after physical deconditioning and exercise training
Immune adaptation to chronic intense exercise training: new	Human gene 1.1 ST array strips (Affymetrix, Inc, Santa Clara, CA)	Venous blood samples	Male and female endurance swimming athletes matched by age,	24 (12 trained and 12 sedentary)	Anti-inflammatory effect of high-intensity endurance training

microarray evidence [32]			sex, and BMI to non-trained individual s		

As per Table 3.2, minimal research has been conducted using microarrays. Of those that have been conducted, studies have small sample sizes (n= 3-24), and muscle biopsies or blood samples were the sample method commonly used. This information can therefore be used to inform the sample size and data analysis of this exploratory research. There are even fewer studies that investigate the use of saliva for microarray kits to investigate cytokine response to sedentary time, this presents a gap within the current research.

In Table 3.3, there is a summary of current research using microarray kits and saliva, with some studies using a combination of saliva, serum, or plasma. The sample size of these studies ranges (n = 5-100), with the median being 10 participants. From these studies we can determine that saliva is a non-invasive option to collect adequate analyte for this study's analysis. As sedentary physiology and the use of array-based methods with saliva has been minimally investigated, this presents an opportunity for this research to fill a void among the current knowledge of sedentary physiology. To date, microarray-based approaches examining exercise impact on cytokines have focused mainly on mRNA to determine the effects on gene expression. By using an antibody-based membrane array to detect a range of cytokines and growth factors in the saliva simultaneously, this research uses a novel approach to investigate the impact on salivary cytokines and growth factors at the protein level. The information reviewed in Tables 3.2 and 3.3 can be used to guide the methods of this thesis.

Table 3.3 Current research using microarray-based methods to analyze genes and gene expression in saliva

Study	Microarray Kit Used	Target	Study Population	Sample Size (n)	Results
Application of microarray and functional-based screening methods for the detection of antimicrobial resistance genes in the microbiomes of healthy humans [33]	Array Mate (Alere Technologies)	Detecting antimicrobial resistance genes in the human gut microbiome (saliva and faecal samples)	Healthy Adults	100 (20 per country)	All samples positive for one or more AMR gene. Six Antibiotic classes were detected.
Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification [34]	miRNeasy Mini Kit	Detecting micro-RNA (miRNA) in saliva samples	Healthy Adults (4 females and 1 males)	5	"We conclude that miRNA extraction from forensic samples is possible and support a "proof of concept" that body-fluid identification by miRNA analysis may become a potent forensic technique. " [34]
Feasibility of the salivary	Affymetrix MicroArray System	Use of salivary transcriptom	10 patients with chronic periodontitis	20	Minimal collection from saliva,

transcriptome as a novel biomarker in determining disease susceptibility [35]		e to measure mRNA (gene expression)	and 10 patients without chronic periodontitis		further research is required.
RNA profiling of cell-free saliva using microarray technology [36]	Affymetrix Human Genome U133A Array	Use of transcriptome to detect mRNA in saliva	Males and females aged 32-55 years old	10	Saliva is a non-invasive and cost-effective diagnostic medium.
MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation [37]	LNA^{TM}-modified oligonucleotides	Salivary miRNA detection	Males and females	6	Additional research required for miRNA markers for saliva.

Table 3.4 summarizes available microarray kits for analysis. For the purpose of this thesis, the specific analytes and cost were important considerations for microarray kit selection. From Table 3.4, the microarray kits found to be most likely to fit the needs of this research include the Proteome Profiler Human Cytokine Array Kit, the Human Cytokine Array C3, and the Abcam Cytokine Array-Human Cytokine Antibody Array. A build your own panel option was considered but required further support from the supplier to determine the kit that would best suit the needs of this research. The Abcam Cytokine Array-Human Cytokine Antibody Array was kit selected to complete this study.

Table 3.4 Antibody-based membrane array kits for human cytokine detection

Microarray kit	Type	Supplier	Cost	Biomarkers of interest
Proteome Profiler Human Cytokine Array Kit	Membrane-based sandwich immunoassay	Bio-Techne R&D Systems	$565/kit	C5a, IL-4, IL-27, CD40 Ligand, IL-5, IL-32 alpha, G-CSF, IL-6, CXCL10/IP-10, GM-CSF, IL-8, CXCL11/I-TAC, CXCL1/GRO alpha, IL-10, CCL2/MCP-1, CCL1/I-309, IL-12 p70, MIF, ICAM-1, IL-13, MIP-1 alpha/MIP-1 beta, IFN-gamma, IL-16, CCL5/RANTES, IL-1 alpha, IL-17, CXCL12/SDF-1, IL-1 beta, IL-17E, Serpin, E1/PAI-1, IL-1ra, IL-18, TNF-alpha, IL-2, IL-21, TREM-1
Proteome Profiler Human XL Cytokine Array Kit	Membrane-based sandwich immunoassay	Bio-Techne R&D Systems	$1, 118/kit	Adiponectin/Acrp30, IFN-gamma, CCL2/MCP-1, Angiogenin, IGFBP-2, CCL7/MCP-3, Angiopoietin-1, IGFBP-3, M-CSF, Angiopoietin-2, IL-1 alpha/IL-1F1, MIF, Apolipoprotein A1, IL-1 beta/IL-1F2, CXCL9/MIG, BAFF/BLyS/TNFSF13B, IL-1ra/IL-1F3, CCL3/CCL4 MIP-1 alpha/beta, BDNF, IL-2, CCL20/MIP-3 alpha, CD14, IL-3, CCL19/MIP-3 beta, CD30, IL-4, MMP-9, CD31/PECAM-1, IL-5, Myeloperoxidase, CD40 Ligand/TNFSF5, IL-6, Osteopontin (OPN), Chitinase 3-like, IL-8, PDGF-AA, Complement Component C5/C5a, IL-10, PDGF-AB/BB, Complement Factor D, IL-11, Pentraxin 3/TSF-14, C-Reactive Protein/CRP, IL-12 p70, CXCL4/PF4, Cripto-1, IL-13, RAGE, Cystatin C, IL-15, CCL5/RANTES, Dkk-1, IL-16, RBP4, DPPIV/CD26, IL-17A, Relaxin-2, EGF, IL-18 Bpa, Resistin, CXCL5/ENA-78, IL-19, CXCL12/SDF-1 alpha,

				Endoglin/CD105, IL-22, Serpin E1/PAI-1, EMMPRIN, IL-23, SHBG, Fas Ligand, IL-24, ST2/IL1 R4, FGF basic, IL-27, CCL17/TARC, KGF/FGF-7, IL-31, TFF3, FGF-19, IL-32 alpha/beta/gamma, TfR, Flt-3 Ligand, IL-33, TGF-alpha, G-CSF, IL-34, Thrombospondin-1, GDF-15, CXCL10/IP-10, TIM-1, GM-CSF, CXCL11/I-TAC, TNF-alpha, CXCL1/GRO alpha, Kallikrein 3/PSA, uPAR, Growth Hormone (GH), Leptin, VCAM-1, HGF, LIF, VEGF, ICAM-1/CD54, Lipocalin-2/NGAL, Vitamin D BP
Human Cytokine Array C1000	Membrane-based sandwich immunoassay	RayBiotech	$650.44/2 sample kit	ADIPOQ, AGRP, ANG, ANGPT2, AREG, AXL, BDNF, BMP4, BMP6, BTC, CCL1, CCL11, CCL13, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL20, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28, CCL3, CCL4, CCL5, CCL7, CCL8, CNTF, CSF1, CSF2, CSF3 , CX3CL1, CXCL1, CXCL11, CXCL12, CXCL13, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL9, EGF, EGFR, FAS, FGF2, FGF4, FGF6, FGF7, FGF9, FIGF, FLT3LG, GDNF, HGF, ICAM1, ICAM3, IFNG, IGF1, IGF1R, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP6, IL10, IL11, IL12A, IL12B, IL13, IL15, IL16, IL17A, IL1A, IL1B, IL1R1, IL1RL1, IL1RN, IL2, IL2RA, IL3, IL4, IL5, IL6, IL6R, IL6ST, IL7, KITLG, LEP, LTA, MIF, MST1, NGF, NTF3, NTF4, OSM, PDGFB, PGF, PLAUR, PPBP, TGFB1, TGFB3, THPO, TIMP1, TIMP2, TNF, TNFRSF10C, TNFRSF10D,

				TNFRSF11B, TNFRSF18, TNFRSF1A, TNFRSF1B, TNFSF14, TNFSF18, TPO, TYRO3, VEGFA, XCL1
Human Cytokine Array C3	Membrane-based sandwich immunoassay	RayBiotech	$440.36/2 sample kit	ANG, CCL1, CCL15, CCL17, CCL2, CCL22, CCL5, CCL7, CCL8, CSF1, CSF2, CSF3, CXCL1, CXCL12, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9, EGF, IFNG, IGF1, IL10, IL12A, IL13, IL15, IL1A, IL1B, IL2, IL3, IL4, IL5, IL6, IL7, KITLG, LEP, LTA, OSM, PDGFB, TGFB1, THPO, TNF, TPO, VEGFA
Human Cytokine Array C5	Membrane-based sandwich immunoassay	RayBiotech	$440.36/2 sample kit	ANG, BDNF, CCL1, CCL11, CCL13, CCL15, CCL17, CCL18, CCL2, CCL20, CCL22, CCL23, CCL24, CCL26, CCL4, CCL5, CCL7, CCL8, CSF1, CSF2, CSF3, CX3CL1, CXCL1, CXCL10, CXCL12, CXCL13, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL9, EGF, FGF4, FGF6, FGF7, FGF9, FLT3LG, GDNF, HGF, IFNG, IGF1, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IL10, IL12A, IL13, IL15, IL16, IL1A, IL1B, IL2, IL3, IL4, IL5, IL6, IL7, KITLG, LEP, LIF, LTA, MIF, NTF3, NTF4, OSM, PDGFB, PGF, PPBP, SPP1, TGFB1, TGFB2, TGFB3, THPO, TIMP1, TIMP2, TNF, TNFRSF11B, TNFSF14, TPO, VEGFA
Cytokine Array-Human Cytokine Antibody Array (Membrane, 42 Targets) (ab133997)	Antibody-pair-based assay	abcam	$616/1x4 membranes	ENA-78, GCSF, GM-CSF, GRO, GRO-alpha, I-309, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IFN-gamma, MCP-1, MCP-2, MCP-3, MCSF, MDC, MIG, MIP-1delta, RANTES, SCF, SDF-1, TARC, TGF-beta1, TNF-alpha, TNF-beta, EGF, IGF-I, Angiogenin, Oncostatin M,

				Thrombopoietin, VEGF-A, PDGF BB, Leptin
Human Cytokine Array Q1	Membrane-based sandwich	Quantibody	$767.20/8 samples	GM-CSF, GRO alpha/beta/gamma, IFN-gamma, IL-1 alpha (IL-1 F1), IL-1 beta (IL-1 F2), IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), MCP-1 (CCL2), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), MMP-9, RANTES (CCL5), TNF alpha, VEGF-A
ProQuantum Immunoassay Kit	N/A	Thermofisher Scientific-Affymetrix	N/A	Pre-built assays or build your own panel option
ProcartaPlex Immunoassays	N/A	Thermofisher Scientific - Luminex	N/A	Inflammation and cytokine storm assays or build your own panel option

Summary: Evidence suggests that biomarkers can be measured using saliva, serum, and plasma samples, but that blood sampling may induce acute inflammation. There are important protocol considerations for saliva sample collection and analysis.

Gaps: This thesis aims to identify which biomarkers respond to prolonged sitting and small bouts of movement to interrupt sitting. It also aims to determine if there are sex dependent variances in biomarker response.

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4. Thesis Rationale, Purpose, and Hypothesis

4.1. Rationale

Research has shown that sedentarism is widespread in society and is associated with physiological disruptions. Currently, our understanding of sedentary physiology is based on our understanding of exercise responses and the physiological benefits seen with exercise training; sedentarism has been linked to vascular dysfunction, metabolic dysfunction, chronic disease risk, and premature death. However, the physiological mechanisms linking sitting time with these disruptions are not apparent. My thesis aims to address this gap by assessing the response of a number of different biomarkers to sitting and sedentary interruptions. Another gap that will be addressed in this work is whether the relevant biomarkers vary by sex.

4.2. Purpose Statement

The purpose of this thesis is to identify which salivary biomarkers respond to prolonged and interrupted sitting sessions in young, healthy males and females, and to identify how responses vary by sex.

4.3. Hypothesis

Given the number of biomarkers being considered in this work, as well as the two conditions, and focus on sex-differences, there are several hypotheses. These hypotheses are summarized in Table 4.1.

Table 4.1 Hypotheses

	Cytokines/Chemokines (IL-8, RANTES, IL-3)	Growth Factors (VEGF, EGF)	Vascular Endothelial Factors (Angiogenin)
Prolonged Sedentary Protocol	Prolonged sedentary time will increase concentrations of IL-8 [1, 2]. Prolonged sedentary time will increase concentrations of RANTES [3]. Prolonged sedentary time will increase concentrations of IL-3 [4].	Prolonged sedentary time will not lead to significant changes in VEGF; however, it will blunt the response of VEGF to exercise [5, 6]. Prolonged sedentary time will increase concentrations of EGF [7-9].	Prolonged sedentary time will decrease concentrations of angiogenin [10, 11].
Interrupted Sedentary Protocol	Sedentary interruptions will decrease concentrations of IL-8 [2, 12]. Sedentary interruptions will decrease concentrations of RANTES [4, 13].	Sedentary interruptions will increase concentrations of VEGF [14-17]. Sedentary interruptions will decrease concentrations of EGF [9, 18].	Sedentary interruptions will increase concentrations of angiogenin [19, 20].
Sex Differences	Females will have higher concentrations of pro-inflammatory cytokines after prolonged sedentary time [21]. Females may have higher levels of pro-inflammatory cytokines compared to males [22].	EGF concentrations may be higher in males or show no sex difference [23, 24]. There will be no significant difference in VEGF concentration in response to interruptions between males and females and no significant difference in VEGF concentrations in males and females at baseline [25].	There may be no sex differences observed or a less profound effect observed in females [10, 20].

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5. Manuscript

Sex-Differences in Novel Biomarkers that Respond to Prolonged and Interrupted Sitting in Healthy Females and Males

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5.1. Abstract

Prolonged bouts of sitting lead to impaired vascular function, impaired metabolic responses, and higher levels of pro-inflammatory markers. Little exploratory work has been conducted to identify biomarkers that respond to prolonged and interrupted sitting. The purpose of this study was to identify novel biomarkers in the saliva (cytokines, growth factors, and vascular factors) that respond to prolonged and interrupted sitting in young, healthy males and females. We also sought to determine sex-differences in these responses. Participants (n = 24, 21.2 years ± 2.2, 50% female) completed a prolonged sitting (PS) session (4 hours without interruption), and an interrupted sitting (IS) session (4 hours with 3 minutes of walking at 60%HRmax every 27 minutes) in random order. Male and female saliva samples were pooled to identify responsive markers using a human cytokine antibody membrane array (42 Targets). Several novel biomarkers were responsive in both sexes (e.g., IL-8, Angiogenin, VEGF, and EGF), in females only (e.g., TNF-α and IL-13), and in males only (e.g., IL-3, RANTES, and IL-12p40/p70). Importantly, several markers were found to respond to prolonged and interrupted sitting (e.g., TNF-α, IL-8, IL-3, RANTES, EGF, Angiogenin, and VEGF). In conclusion, prolonged sitting appears to increase concentrations of some pro-inflammatory cytokines and growth factors. There are sex-differences in the biomarkers that can be identified in the saliva, and that respond to prolonged and interrupted sitting.

Keywords: Sedentary, cytokines, growth factors, endothelial function, exercise.

5.2. Introduction

Sedentary behaviour is defined as any waking behaviour that has an energy expenditure of ≤ 1.5 metabolic equivalents, while in a sitting, reclining, or lying posture [1]. The prevalence of sedentary behaviour is high, with Canadians spending 69% of their waking hours engaging in sedentary pursuits [2]. Accumulating high volumes of sedentary time is associated with increased risk of mortality [3-6], cardiovascular disease, and metabolic diseases such as diabetes [7-11]. It has also been show to negatively affect cardiometabolic biomarkers [6], vascular function [12-15] and pro-inflammatory biomarkers [16, 17]. There is some evidence to suggest that even ***short bouts of prolonged sitting*** are associated with physiological disruptions.

The physiological responses to acute bouts of sitting have primarily been studied using cardiometabolic markers [18-20]. Systematic reviews of such studies have shown that prolonged sitting is adversely associated with cardiometabolic markers such as plasma glucose, C-reactive protein (CRP), insulin sensitivity, and lipids, and that lower limb vascular function, as measured by lower-limb flow mediated dilation (FMD) and shear rate, is progressively impaired as a consequence of prolonged sitting [21, 22]. Importantly, it has been consistently noted that interrupting sedentary time with exercise attenuates or abolishes the effects of prolonged sitting on these outcomes [23-25]. Peddie et al., investigated the effect of three protocols, prolonged sitting, prolonged standing, and prolonged sitting with exercise interruption (2 minutes of treadmill walking at 5 km/h; 10% incline) on vascular, glucose, and insulin responses (n = 18, age: 23.5 years ± 5.0). Exercise interruptions were found to increase blood flow and net shear rate compared to prolonged sitting; prolonged standing also led to initial improvements in blood flow, however it was not maintained throughout the six-hour protocol. Exercise interruptions also led to a reduction in postprandial insulin when compared to prolonged sitting and standing [26]. Thus, while it is clear that ***interrupting prolonged sitting with exercise*** of even a light intensity can lead to improved cardiometabolic responses, little work has been done to understand the response of pro-inflammatory markers or vascular factors.

Two studies from our laboratory investigated the acute response of Interleukin (IL)-8, a pro-inflammatory cytokine. In both, a prolonged sitting and sitting interrupted with

exercise session was completed by healthy males and females [27, 28]. Analysis of saliva samples indicated that IL-8 levels increased in response to prolonged sitting; however, ***the response to the exercise interruptions varied*** such that the light intensity interruption only blunted the response while the high intensity interruption abolished the response. Importantly, for the light intensity interruptions, when the data were compared between males and females, interrupting sitting appeared to abolish the increase in IL-8 among males, and did not appear to have any impact on the IL-8 response in females [28]. This is an important ***sex-difference*** as it points to either a need for different interruption doses or perhaps investigation of different biomarkers. Previous research has indicated that such sex-differences may also exist for markers such as triglycerides, insulin sensitivity, glucose metabolism, vascular function (flow mediated dilation), and biomarkers like IL-6, IL-8, Fibrinogen and RANTES [18, 28-31]; however, little work in the area of sedentary physiology has adequately addressed such sex-differences in response to prolonged and interrupted sitting.

Evidence shows that acute bouts of prolonged sitting leads to physiological disruptions that interruptions with exercise can attenuate or even abolish. Given the volume of sedentary time we are engaging in, and the negative health consequences of sedentary time, research is needed to better understand the physiological responses to prolonged sitting, and to light intensity exercise interruptions. While it is clear that cardiometabolic markers such as glucose are responsive to prolonged and interrupted sitting, our understanding of other biomarkers is limited. Thus, the primary purpose of this study was to identify novel biomarkers that respond to periods of prolonged and interrupted sitting. We hypothesize that pro-inflammatory biomarkers, including myokines and chemokines, will increase in concentration with prolonged sitting and this increase will be blunted or abolished with exercise interruption, based on previous research from our lab [27, 28, 32], the response of myokines to exercise [33, 34], and the established role of chemokines in the inflammatory response [35]. Given the lack of research on sex-differences on the response to prolonged and interrupted sitting, a secondary purpose of this study was to determine whether biomarker responses to prolonged and interrupted sitting would differ by sex. We hypothesized that females would have a more pro-inflammatory biomarker profile at baseline and would have a less profound response to exercise interruption than males, based

on previously recorded increased immune response and increased incidence of auto-immune disease among females [18, 28, 29, 36-38].

5.3. Methods

Study Design: A randomized crossover design was used. Participants were randomly allocated to complete either the Prolonged Sitting (PS) session or the Interrupted Sitting (IS) session first using a number generator, with balanced randomization. Sessions took place at least one week apart.

Participants: Eligible participants were males and females between the ages of 18-30 years, with a body mass index (BMI) of <30 kg/m^2, who were non-smokers. Individuals were excluded if they had an existing respiratory, cardiovascular, or metabolic condition, an acute infection, were pregnant, were taking any medication that would influence their inflammatory or exercise response, had an acute injury, or had recent dental surgery. All participants provided written informed consent prior to participation in the study. This study was approved by the Research Ethics Board at Ontario Tech University (REB #16473).

Due to the exploratory and novel nature of this research, as well as the sex-based pooled analysis approach, a sample size and power calculation were not possible as there was no effect size. Based on previous literature of exercise and the use of microarrays [39-41] and saliva and the use of microarrays [27, 42, 43], which vary in sample size of n=10-24, median 14, and n=10, median 10, respectively, we chose a sample size of 24 participants.

Protocols: Participants completed three laboratory sessions: 1) baseline session, 2) PS, and 3) IS. The latter two were conducted in random order. Data collection was conducted from October 2021 to February 2022. For the PS, participants were seated continuously for four hours. For the IS, the four hours prolonged session was interrupted with three-minute activity interruptions (60% Heart Rate (HR)max) every 27 minutes (at times: 27, 57, 87, 117, 147, 177, 207, and 237 minutes), for a total of eight interruptions. The four hour duration was chosen as it best mimics a typical sitting pattern. Participants were instructed to perform as little lower limb movement as possible, however, they were permitted to use their upper limbs for the study duration. Participants were instructed to arrive fasted and

were provided a standardized breakfast and snack during the sessions (590 Calories; Fats: 6.5g, Carbohydrates: 127g, Proteins: 11.1g).

Baseline and Maximal Exercise Measures: Resting HR, resting blood pressure (A&D Medical Digital Blood Pressure Monitor, Model UA-767FAM, A&D Engineering, Inc. San Jose, CA, USA), height (cm), and body mass (kg) (Detecto Weight Beam Eye-Level, Webb City Missouri) were measured.

An incremental to maximal exercise test using a stepwise protocol was performed on a treadmill (Trackmaster, FullVision, Newton, KS). Participants were fitted with a portable HR monitor (Polar Electro Oy, Professorintie 5, FI-90440 Kempele, Finland) for continuous measurement of HR. A metabolic cart was used for breath-by-breath gas analysis (Parvo Medics 2400, USA). Concentrations of expired O_2 and CO_2 were analysed, and ventilation was measured. Test termination criteria included a Respiratory Exchange Ratio (RER) >1.15, HR ± 10 beats per minute of age-predicated maximal HR (220-age), a plateau in oxygen uptake (VO_2), or volitional fatigue. The highest HR recorded during the test was used for HRmax. VO_2max was calculated as the highest VO_2 that was attained during the test. After finding the peak VO_2, VO_2max was calculated as a mean of ±5 breaths, including this value.

Saliva Collection and Analysis: Saliva samples were taken upon arrival at each of the three sessions (T1) and at the end of the PS and IS sessions (T2). Participants were instructed to refrain from alcohol, smoking, strenuous exercise, and the use of anti-inflammatory medications in the 24-hours prior to the session. They were also instructed to arrive fasted (overnight fast), refrain from caffeine/stimulants, supplements, or mouthwash, and were instructed to attempt to drink 1L of water on the day of the session.

Saliva samples were collected using oral swabs (Salimetrics SalivaBio, Salimetrics LLC, State College, PA, USA). The swab was placed under the tongue or against the cheek for five minutes and then immediately centrifuged (VWR Clinical 2000, Germany) at 4000 rpm for five minutes allowing collection of 1.5-3.0 mL of saliva, which was subsequently stored at -80 °C. Samples were thawed on the day of analysis and were centrifuged for 15 minutes at 1500 x g at 4 °C to remove mucins and particulate matter that could potentially

interfere with antibody binding, then pooled into male and female subgroups of: PS-T1, PS-T2, IS-T1, and IS-T2.

Total salivary protein concentrations of pooled male and female samples were determined using the Coomassie PLUS 138 Protein Assay Reagent (Thermo Fisher Scientific, MA, USA) prior to sample analyses. Cytokines, including several chemokines, growth factors and vascular/endothelial factors within the saliva samples were quantified using Human Cytokine Antibody Array Membranes following manufacturer's protocols (Abcam, Catalog # ab133997). The full list of biomarkers analyzed by the microarray kit and their alternate names are presented in **Table 5.1**. Briefly, 250 µg of total protein, allowing for standardization across samples, from either the male or female pooled samples from each cohort were loaded onto the membranes and subsequently incubated overnight at 4 °C under gentle rotation. Following the overnight incubation, an extra wash was performed prior to loading the biotin-conjugated anti-cytokine antibody onto the membranes. Next, a wash was performed and the HRP (Horseradish Peroxidase)-conjugated Streptavidin was added to the membrane and incubated for 2-hours at room temperature. Lastly, a detection buffer containing HRP substrate was added and incubated for 2-minutes at room temperature and then chemiluminescence was measured. Chemiluminescence detection and qualitative determination of cytokine expression within the different samples was performed using a LiCor C-DiGit® Blot Scanner and Image Studio™ imaging software. To calculate relative cytokine expression levels, the summed signal density of each spot was background corrected and normalized to the positive controls across all membranes.

Statistical Analysis: Descriptive statistics (Means ± SD) were performed on sample characteristics. Human cytokine antibody array results were compiled into a table and graphs to visually compare female and male data from the PS and IS sessions at T1 and T2. String Diagram generator was used to demonstrate relationships between select responsive and detectable cytokines and growth factors [44].

5.4. Results

Participant characteristics (n = 24, mean 21.1 years ± 2.2, 50% female) can be found in **Table 5.2**.

Several cytokines and growth factors were not present at detectable levels in the saliva (IL-2, MCP-2, TNF-β, MCP-3, IL-5, IL-6, IL-7, MIP-1δ, IL-10, I-309, IL-1α, TARC, IFN-γ, G-CSF, GM-CSF, and Leptin). Some cytokines and growth factors were only detectable in females (TNF-α, Thrombopoietin (only IST2), and IL-13 (only PST2)), while some cytokines and growth factors were only detectable in males (IL-3, IL-4 (only IST2), GRO, RANTES, GRO-α/CXCL-1, IL-12, SCF, PDGF-BB, and SDF-1/CXCL-12).

Mean spot pixel densities, indicating relative levels of detectable cytokines and growth factors in saliva, from PS and IS at T1 and T2 are presented in **Table 5.3**. Interestingly, differences in IL-3 (**Figure 5.1**) indicate that some cytokines are detectable in the saliva in males but not in females. For example, among males, it appears that levels of IL-3 remained the same during PS (T1 = 161.0; and T2 = 142.8) but were higher from T1 to T2 during IS (T1 = 139.4; and T2 = 321.0).

Patterns among relative levels of salivary EGF, VEGF, and Angiogenin also provide interesting insight (**Figures 5.2-5.4**). Data on EGF (**Figure 5.2**), indicate that among females, EGF levels appear to be lower from T1 to T2 during PS (T1 = 1023.4; and T2 = 369.0) and IS (T1 = 955.0; and T2 = 503.2). Among males, the opposite pattern was observed, with EGF appearing to be higher from T1 to T2 during PS (T1 = 12728.5; and T2 = 26622.2) and IS (T1 = 22636.8; and T2 = 34446.0). Analysis of VEGF (**Figure 5.3**) indicates a similar pattern to EGF between males and females. Among females, relative VEGF levels appear to be lower from T1 to T2 during PS (T1 = 703.6; and T2 = 431.6) and remain the same during IS (T1 = 659.0; and T2 = 663.1). Among males, VEGF appears to be higher from T1 to T2 during PS (T1 = 197.0; and T2 = 423.5) and IS (T1 = 469.8; and T2 = 758.1). Data on Angiogenin (**Figure 5.4**) indicate a similar pattern between males and females. Among females, Angiogenin levels appear to be higher from T1 to T2 during PS (T1 = 69.8; and T2 = 459.7) and IS (T1 = 58.1; and T2 = 538.6). Among males, Angiogenin also appears to be higher from T1 to T2 during PS (T1 = 310.5; and T2 = 6876.5) and IS (T1 = 272.5; and T2 = 6994.6).

Relationships between responsive and detectable cytokines and growth factors were assessed using the String Diagram generator [44]. First, the nine biomarkers that indicated sex-differences were inputted (**Figure 5.5**). Associations were determined between eight of the nine cytokines and growth factors. Second, the five biomarkers with observed changes between the PS and IS conditions were inputted (**Figure 5.6**). Associations were determined between all five cytokines and growth factors.

5.5. Discussion

We sought to identify novel biomarkers that responded to prolonged and interrupted bouts of sitting and to determine if biomarker response varied dependent on sex. Our primary finding is that four hours of prolonged sitting led to changes in concentrations of several cytokines, vascular factors, and growth factors in the saliva. Our secondary finding is that biomarkers varied in presence in saliva, response to prolonged sitting, and response to sedentary interruptions dependent on sex. These findings provide interesting insights into the role of salivary biomarkers and sex in sedentary physiology and the intensity of exercise required to elicit a response from specific biomarkers.

We hypothesized that **pro-inflammatory markers** would increase in concentration in response to prolonged sitting, and that this increase would be blunted by an interruption protocol. This trend was observed in EGF, IGF-1, and GRO-α for males and in TGF-β1, TNF-α, and IL-8 for females. EGF appeared to be higher from baseline to the end of the 4-hour prolonged sitting session and appeared to have a less profound increase with the interruptions in males. This observation was supported by previous research that shows that EGF increases with prolonged sitting [45-47], decreases with exercise [45, 48], and that there may be differences between the sexes in the effects of exercise on EGF [49, 50]. For IGF-1 and TGF-β1, the changes were not as expected. IGF-1 appeared to be higher from baseline to the end of the 4-hour prolonged sitting session and this increase was attenuated with interruptions for males, but not for females. This was surprising since previous research states that IGF would increase with exercise [51]. TGF-β1, is considered regulatory rather than solely pro-inflammatory and has been shown to increase with acute exercise due its role as a myokine [52], which is the opposite of what was observed in this study, as exercise appeared to blunt the increase of TGF-β1. This could be explained by

the moderate intensity of exercise used; the intensity of exercise used in this protocol may not have been sufficient to elicit an increase of IGF and TGF-β1 or the variation could be attributed to the different cell and tissue targets, and the presence of and interactions with other biomarkers. Plasma and serum samples have been documented as having higher concentrations of TNF-α with prolonged sedentary time [53, 54], and due to its pro-inflammatory designation, decreasing with exercise [55]. This is supported among females in our results, where TNF-α appeared to be higher from baseline to the end of the 4-hour prolonged sitting session and showed an attenuation of this increase with interruptions. Thus, overall, we found that of the pro-inflammatory markers from the saliva that responded, the general trend was as hypothesized, this is depicted visually in the String Diagram (**Figure 6**). However, there are some noteworthy differences in the response to exercise that require further investigation into the intensity of interruptions.

We hypothesized that **myokines** such as IL-8, IL-6, IL-15 and TNF-α would be the most responsive to prolonged and interrupted sitting given that they respond to muscular contraction [33, 34]. Among females in our sample, TNF-α and IL-8 responded as anticipated. This is in line with previous research that has found an increase in concentrations of IL-8 mRNA and protein expression in the skeletal muscle in response to exercise [56, 57]. Among males, TNF-α was not observed, while IL-8 concentrations appeared to be lower from baseline to the end of the 4-hour prolonged sitting protocol and higher from baseline to the end of the 4-hour interrupted sitting protocol, indicating that IL-8 as a myokine is responsive to sedentary interruptions with moderate intensity exercise. IL-15 has previously been shown to increase after exercise [58], this also supports that myokines are responsive to sedentary interruptions. In keeping with our results, among males we observed an increase from baseline to the end of the 4-hour prolonged sitting session, and a greater increase from baseline to the end of the 4-hour interrupted sitting session. Thus overall, we observed that myokines responded as hypothesized to exercise interruption.

We hypothesized that **chemokines** such as MCP-1 and RANTES would be responsive to prolonged and interrupted sitting based on the established role of chemokines in the inflammatory response to prolonged sitting [27, 28, 59]. IL-8, GRO-α, and MCP-1were

found to be responsive to prolonged and interrupted sitting. Among females, IL-8 and MCP-1 appeared to be higher from baseline to the end of the 4-hour prolonged sitting protocol, IL-8 had a less profound increase in the interrupted sitting protocol, whereas MCP-1 had a more profound increase in the interrupted sitting protocol. This is supported by previous research from our lab documenting the increase of salivary IL-8 levels with prolonged sitting and an attenuation of this increase with exercise interruption [28]; and from other studies stating that serum levels of MCP-1 increase post-exercise [30]. Interestingly, among the males in our sample, there was significant variation in the response of pro-inflammatory markers, such as IL-8, RANTES, MCP-1, and GRO-α, which have previously been shown to increase in response to prolonged sitting and to be responsive to exercise interruptions [27, 30, 32, 53]. In our study IL-8 and RANTES appeared to be lower from baseline to the end of the 4-hour prolonged sitting protocol and higher from baseline to the end of the 4-hour interrupted sitting protocol, MCP-1 appeared to be higher from baseline to the end of the prolonged sitting protocol and was not observed in the interrupted sitting protocol, and GRO-α appeared to be higher from baseline to the end of the 4-hour prolonged sitting protocol with a less profound increase in the interrupted sitting protocol. This is contrary to previous research which has documented GRO as increasing post-exercise [30] and IL-8 increasing with prolonged sitting and this increase being attenuated or abolished with exercise [27, 28]. One of the reasons for this discrepancy could be the intensity of the exercise interruption. It is possible that some biomarkers would need a greater intensity, such as IL-8, as indicated in the introduction [27, 28] or some biomarkers would need a lighter intensity, such as GRO-α, to avoid the acute stress produced by exercise [60]. Overall, chemokines followed the hypothesized trend of responding to prolonged and interrupted sitting protocols.

We hypothesized that **growth and endothelial factors** would be responsive to a prolonged and interrupted sitting protocol. We observed that certain growth and endothelial factors were responsive to the two protocols and have demonstrated consistency between the sexes, specifically Angiogenin. Angiogenin appeared to be higher from baseline to the end of the 4-hour prolonged sitting protocol and to have a more profound increase in the interrupted sitting protocol in both the male and female sample. This is supported by previous research, which states that Angiogenin concentrations will increase with exercise

and that there may be no sex differences observed [61, 62]. Yet, there was large variation in the response of other growth and endothelial factors, specifically, IGF-1, EGF, and VEGF, which showed variation or unanticipated responses between sexes and protocols. Further research may be needed to investigate the role of prolonged sitting and exercise interruption on biomarkers of vascular function and growth factors in regard to exercise intensity, duration, and potential sex differences.

We hypothesised that one, there would be **sex-differences** in the response to prolonged and interrupted bouts of sitting, and two, that females would have higher concentrations of pro-inflammatory biomarkers at baseline. Our results support the trend that there are sex-differences in the response of biomarkers to prolonged and interrupted bouts of sitting, such as IL-8, TNF-α, EGF, and IGF-1 varying in their response between males and females, as illustrated in the String Diagram in **Figure 5.6**. The discrepancy in responses of males and females to the prolonged and interrupted sitting protocols could be attributed to their established differences in response to exercise [63]. The lower number of pro-inflammatory biomarkers present after prolonged sitting among females could be supported by their less profound response to prolonged sitting documented by Restaino et al. [18]; this could influence the number of pro-inflammatory biomarkers detectable in saliva pre and post prolonged sitting. Contrary to previous research, suggesting that females have higher concentrations of pro-inflammatory biomarkers and more active immune systems [36, 38, 64-67], we did not observe higher concentrations of pro-inflammatory markers in females. Instead, males were observed to have both a higher number of total biomarkers and a higher number of pro-inflammatory biomarkers detectable at baseline in saliva. Males and females were observed to have similar anti-inflammatory biomarkers at baseline. Further research may be needed to investigate the sex-specific responses of biomarkers to prolonged sitting and the role of exercise intensity on sex-dependent responses.

To our knowledge, this is the first study to investigate a large number of biomarkers present in saliva, with the use of an antibody array kit, in the context of sedentary physiology. Previous research in the field has focused on a few well-known cytokines [68], with limited research into growth and endothelial factors. We identified eight growth and

endothelial factors as well as eighteen cytokines in the saliva of healthy males and females. Thus, our work provides further support for analysis of saliva, a non-invasive and simple method that can be applied across different physiological studies, including sedentary and exercise [69, 70]. The use of the bioinformatic tool, String Diagram generator [44], allowed for the determination of several relationships between the detected biomarkers, such as experimental determination of protein-protein interactions, co-expression, and significant protein interactions as documented in scientific literature. The use of this tool could help to identify which biomarkers present a gap in the research and help to guide future studies to select a biomarker for investigation. Another strength of the current study is the homogenous sample of young, healthy individuals and the randomized cross-over design. Some limitations include the lack of individual level analysis and the lack of blood samples for comparison. The timing of the post-protocol saliva sample may have also limited the detection of biomarkers. Previous literature has varied in their timing of collection of biomarkers, typically occurring immediately post-intervention, 2-hours post-intervention, or 24-hours post-intervention [28, 32, 71-73]. A salivary sample collection 2-hour post-protocol or 24-hour post-protocol may have allowed for the detection of other biomarkers or biomarkers to be collected at their peak of activity. Future research should consider having multiple interruption sessions with different exercise intensities for comparison. Future work should also investigate how these factors respond at an individual level, the potential mechanisms responsible for these responses, the role of intensity on biomarker response to exercise, the effect of collection timing on biomarker response, and the effect of sex on biomarker response.

In conclusion, four hours of prolonged sitting lead to a change in salivary biomarker concentrations that varied dependent on sex and when the prolonged sitting was interrupted with regular, moderate-intensity exercise. Additionally, several growth and endothelial factors responded to prolonged and interrupted sitting protocols. Future work should investigate how these factors respond at an individual level, to different exercise intensity interruptions, and the role of sex in biomarker response.

5.6. References

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Table 5.1 Biomarkers investigated using the Abcam Human Cytokine Antibody Array Membranes

Name	Alternate Name(s)
Cytokine	
Interleukin-3 (IL-3)	Colony-Stimulating Factor (CSF) Multi-CSF (MULTI-CSF) Mast Cell Growth Factor (MCGF)
Tumour Necrosis Factor-Beta (TNF-β)	
Interleukin-4 (IL-4)	B-cell Stimulatory Factor-1 (BSF-1)
Interleukin-5 (IL-5)	T-cell Replacing Factor (TRF) Eosinophil Differentiation Factor (EDF) B-cell Differentiation Factor
Macrophage Colony Stimulating Factor (MCSF)	
Oncostatin M (OSM)	
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	Colony Stimulating Factor 2 (CSF2)
Thrombopoietin (THPO)	Megakaryocyte Growth and Development Factor (MGDF)
Interleukin-10 (IL-10)	Human Cytokine Synthesis Inhibitory Factor (CSIF)
IL-12 p40/p70	Cytotoxic Lymphocyte Maturation Factor (CLMF) NK Cell Stimulatory Factor Chain (NKSF)
Stem Cell Factor (SCF)	KIT-Ligand (KL) Steel Factor
Interleukin-1-Alpha (IL-1α)	Fibroblast-Activating Factor (FAF) Lymphocyte-Activating Factor (LAF) B-cell Activating Factor (BAF) Endogenous Pyrogen Catabolin Hemopoietin-1 (H-1) Melanoma Growth Inhibition Factor (MGIF) Osteoclast Activating Factor
Interleukin-1-Beta (IL-1β)	Leukocyte Pyrogen Leukocytic Endogenous Mediator

	Mononuclear Cell Factor Lymphocyte Activating Factor
Interferon-Gamma (IFN-γ)	Type II Interferon
Leptin	Peptide Hormone (Obesity Factor)
Chemokine	
Monocyte Chemoattractant Protein-1 (MCP-1)	CCL2
Interleukin-2 (IL-2)	
Monocyte Chemoattractant Protein-2 (MCP-2)	CCL8
Monocyte Chemoattractant Protein-3 (MCP-3)	CCL7
Epithelial Neutrophil-Activating Peptide (ENA-78)	CXCL5
Macrophage Derived Chemokine (MDC)	CCL22
Monokine Induced by Interferon Gamma (MIG)	CXCL9
Interleukin (IL-8)	CXCL8 T Cell Chemotactic Factor Neutrophil-Activity Peptide 1 Beta-thromboglobulin-like protein
Macrophage Inflammatory Protein 1 Delta (MIP-1δ)	CCL15 Leukoactin-1 (LKN-1)
Growth-Regulated Oncogene (GRO)	CXCL1 (GRO-α) CXCL 2 (GRO-β) CXCL 3 (GRO-γ)
Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES)	CCL5
Growth-Regulated Oncogene-Alpha (GRO-α)	CXCL1 Neutrophil-Activating Protein 3 (NAP-3) Melanoma Growth Stimulating Activity, Alpha (MGSA-α)
I-309	CCL1
Stromal Cell-Derived Factor (SDF-1)	CXCL12
Thymus and Activation Regulated Chemokine (TARC)	CCL17
Myokine	
Tumour Necrosis Factor-Alpha (TNF-α)	TNF Cachexin
Interleukin-6 (IL-6)	B-cell Stimulatory Factor 2 (BSF-2) CTL Differentiation Factor (CDF) Hybridoma Growth Factor Interferon beta-2 (IFN-beta-2)
Interleukin-7 (IL-7)	Lymphopoietin 1 (LP-1)

	Pre-B Cell Factor
Interleukin-13 (IL-13)	
Interleukin-15 (IL-15)	
Growth/Vascular/Endothelial Factor	
Epidermal Growth Factor (EGF)	
Insulin-like Growth Factor-1 (IGF-1)	Somatomedin C
Angiogenin (ANG)	Ribonuclease 5
Granulocyte-Colony Stimulating Factor (GCSF)	Colony Stimulating Factor 3 (CSF 3)
Vascular Endothelial Growth Factor (VEGF)	Vascular Permeability Factor (VPF)
Platelet Derived Growth Factor (PDGF) BB	
Transforming Growth Factor-Beta-1 (TGF-β1)	

Table 5.2 Participant characteristics

	Female (n = 12)	Male (n = 12)
Age (years)	20.9 ± 1.8	21.3 ± 2.5
Body Mass Index (kg / m^2)	24.4 ± 2.1	25 ± 3.6
Physical Activity (minutes · week $^{-1}$)	154.2 ± 74.9	158.8 ± 189.6
Maximal Aerobic Capacity – VO_{2max} (mL · kg^{-1} · min^{-2})	37.2 ± 4.9	45.6 ± 8.5

Table 5.3 Average Spot Pixel Density of Cytokines (a) and Growth and Vascular/Endothelial Factors (b) to Prolonged and Interrupted Sitting Sessions at T1 and T2 in females (n=12) and males (n=12)

a)

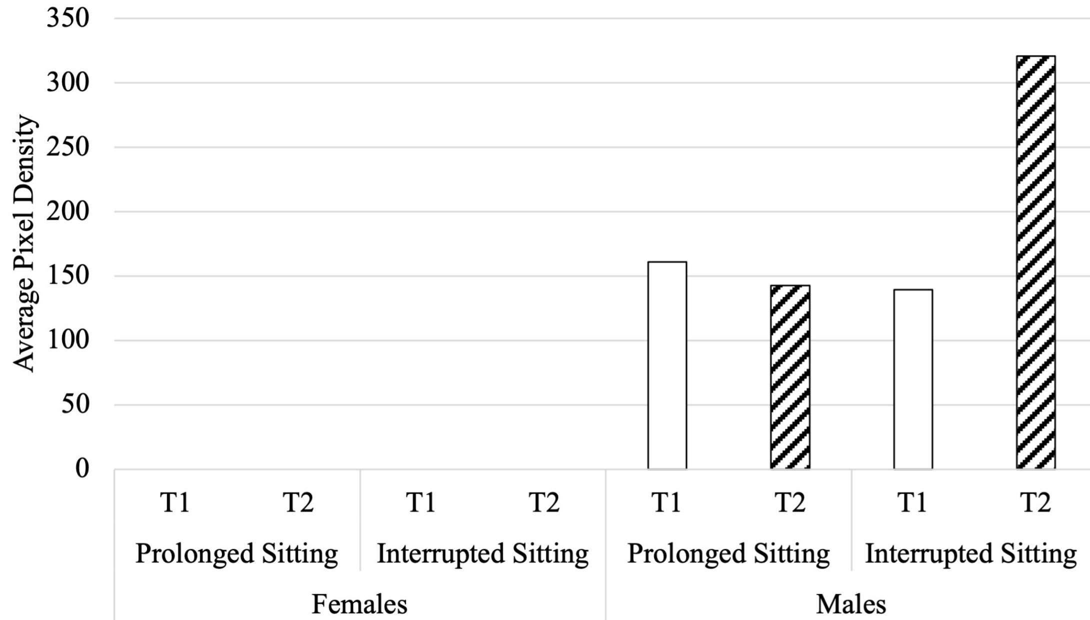
Cytokine	Females				Males			
	Prolonged Sitting		**Interrupted Sitting**		**Prolonged Sitting**		**Interrupted Sitting**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
MCP-1	415.3	877.8	407.4	3996.6	0.0	176.0	0.0	0.0
TNF-α	0.0	231.2	0.0	119.8	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	161.0	142.8	139.4	321.0
IL-4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	137.6
MCSF	0.0	0.0	0.0	95.9	0.0	168.8	116.0	355.2
ENA-78	0.0	0.0	0.0	68.4	0.0	0.0	0.0	389.3
MDC	179.8	252.2	188.3	91.7	167.5	135.0	0.0	171.8
MIG	0.0	0.0	0.0	234.9	57.5	106.3	0.0	0.0
Oncostatin M	0.0	0.0	0.0	128.6	0.0	207.5	166.7	416.6
IL-8	284.2	598.4	298.3	475.2	323.5	305.5	454.3	471.3
Thrombopoietin	0.0	0.0	0.0	119.0	0.0	0.0	0.0	0.0
GRO	0.0	0.0	0.0	0.0	0.0	98.2	0.0	286.4
RANTES	0.0	0.0	0.0	0.0	103.0	0.0	170.1	252.2
GRO-α	0.0	0.0	0.0	0.0	0.0	136.5	113.8	225.9
IL-12 p40/p70	0.0	0.0	0.0	0.0	133.0	134.1	99.2	198.1
IL-13	0.0	394.3	0.0	0.0	0.0	0.0	0.0	0.0
IL-15	0.0	0.0	0.0	85.1	0.0	105.3	0.0	179.1
IL-1β	0.0	0.0	0.0	107.7	361.0	203.5	326.3	508.8

b)

Growth Factor/Endothelial Factor	Females				Males			
	Prolonged Sitting		**Interrupted Sitting**		**Prolonged Sitting**		**Interrupted Sitting**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
EGF	1023.4	369.0	955.0	503.2	12728.5	26622.2	22636.8	34446.0
IGF-1	335.2	274.7	347.4	251.4	145.4	378.2	519.1	676.6
Angiogenin	69.8	459.7	58.1	538.6	310.5	6876.5	272.5	6994.6
VEGF	703.6	431.6	659.0	663.1	197.0	423.5	469.8	758.1

SCF	0.0	0.0	0.0	0.0	95.2	116.3	0.0	163.0
PDGF BB	0.0	0.0	0.0	0.0	0.0	97.4	0.0	215.2
SDF-1	0.0	0.0	0.0	0.0	885.0	699.1	650.4	732.2
TGF-β1	0.0	635.8	0.0	393.0	0.0	108.6	0.0	173.7

Figure 5.1 Average Pixel Density of IL-3 Before (T1) and After (T2) Prolonged and Interrupted Sitting Sessions in Females (n=12) and Males (n=12)

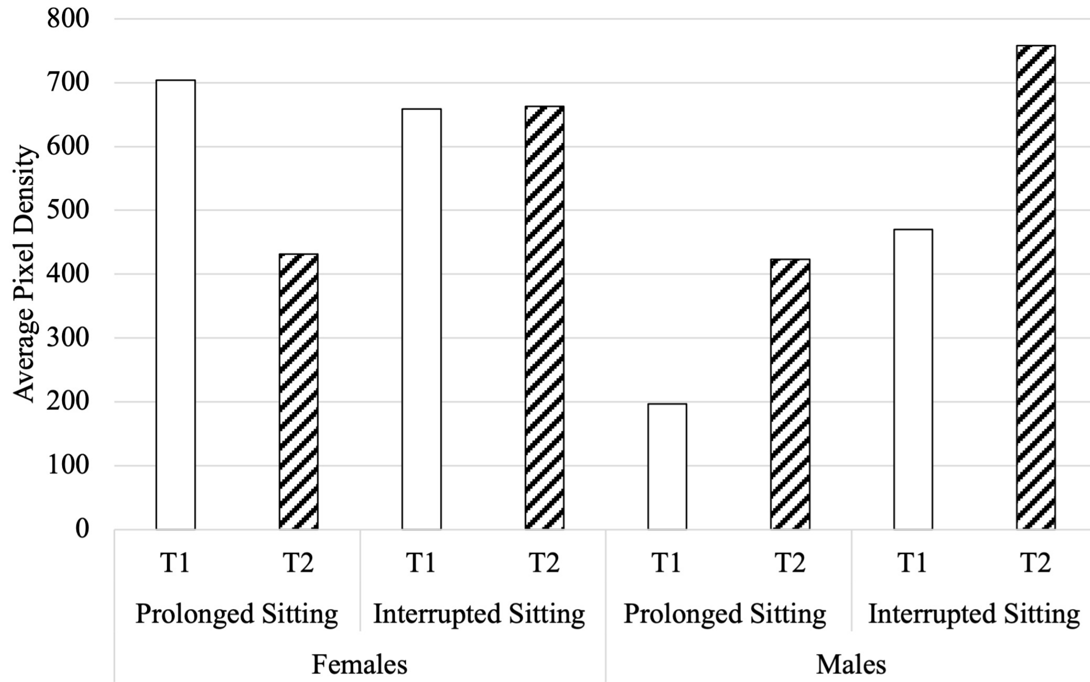


Average pixel density of salivary IL-3 from prolonged and interrupted sitting sessions at T1 and T2 are reported by sex. IL-3 was not detected in the saliva of females. In males, IL-3 appears to show little change from T1 to T2 in the prolonged sitting session and appears to increase from T1 to T2 in the interrupted sitting session.

Figure 5.2 Average Pixel Density of EGF Before (T1) and After (T2) Prolonged and Interrupted Sitting Sessions in Females (n=12) and Males (n=12)

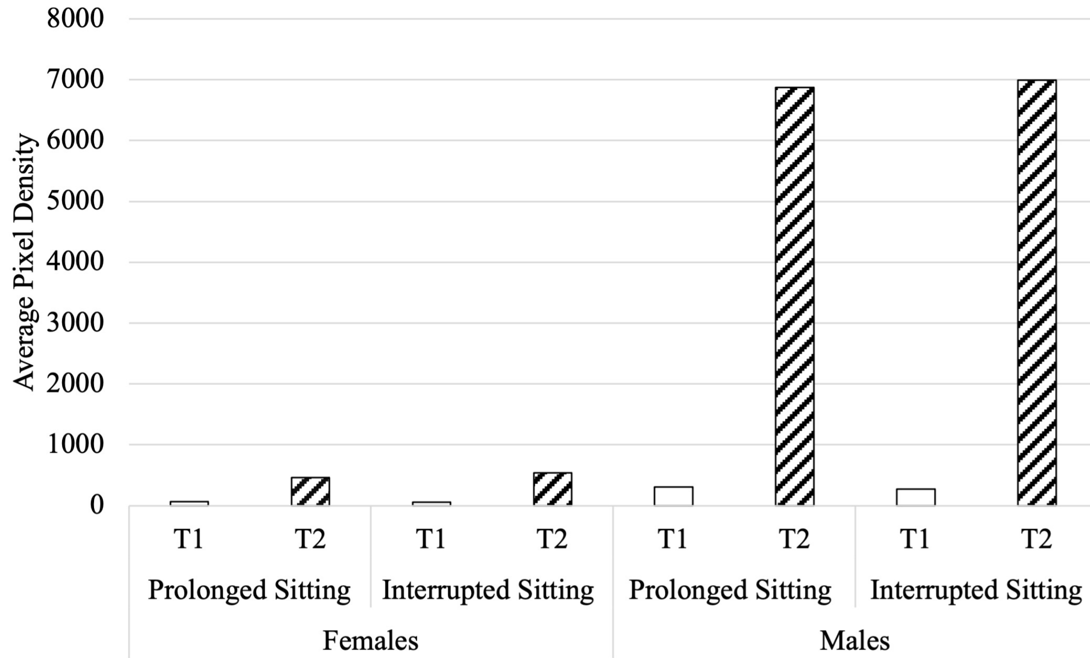
Average pixel density of salivary EGF from prolonged and interrupted sitting sessions at T1 and T2 are reported by sex. Among females, EGF appears to decrease from T1 to T2 in both conditions. Among males, EGF appears to increase from T1 to T2 in both conditions.

Figure 5.3 Average Pixel Density of VEGF Before (T1) and After (T2) Prolonged and Interrupted Sitting Sessions in Females (n=12) and Males (n=12)



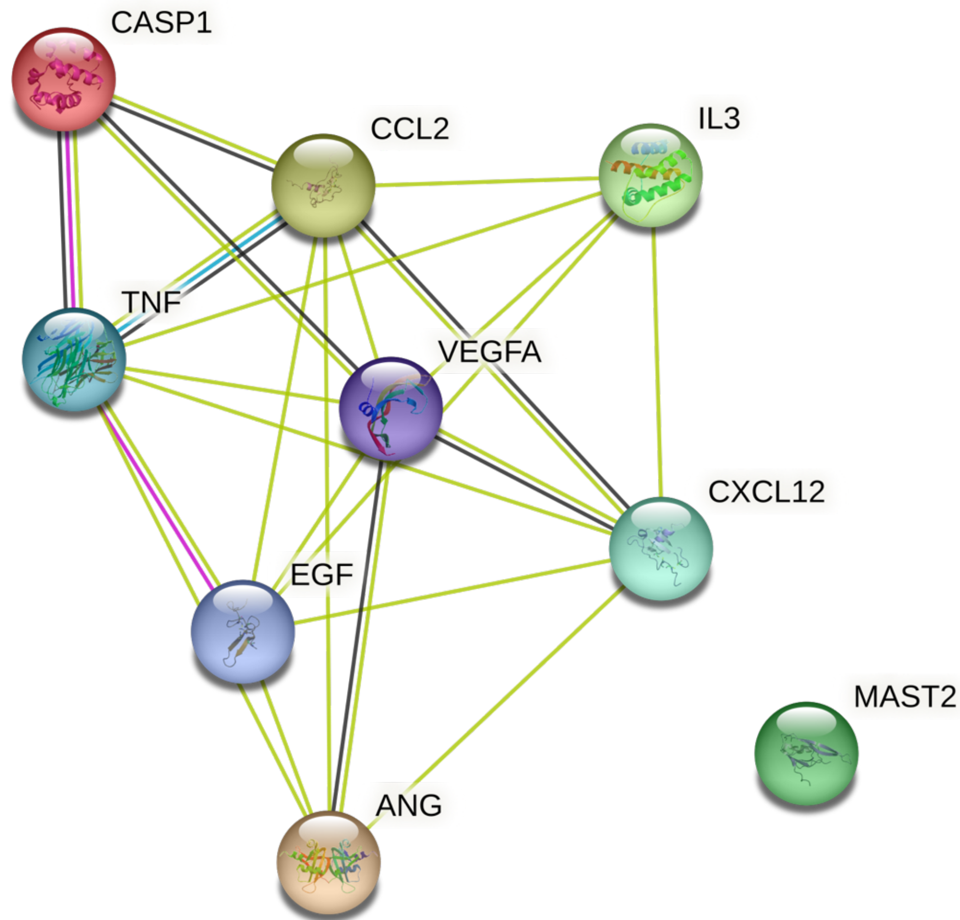
Average pixel density of salivary VEGF from prolonged and interrupted sitting sessions at T1 and T2 are reported by sex. Among females, VEGF appears to decrease from T1 to T2 in the prolonged sitting session but showed minimal change from T1 to T2 in the interrupted sitting session. Among males, VEGF appears to increase from T1 to T2 in both conditions.

Figure 5.4 Average Pixel Density of Angiogenin Before (T1) and After (T2) Prolonged and Interrupted Sitting Sessions in Females (n=12) and Males (n=12)



Average pixel density of salivary Angiogenin from prolonged and interrupted sitting sessions at T1 and T2 are reported by sex. Among females and males, Angiogenin appears to increase from T1 to T2 in both conditions.

Figure 5.5 Physiological Pathways and Connections between Cytokines and Growth Factors that Demonstrated Sex Differences



Connections between nine cytokines and growth factors that demonstrated some sex differences are displayed using a String Diagram. CASP1 (IL-1β), IL-3, TNF (TNF-α), VEGFA (VEGF), CCL2 (MCP-1), EGF, ANG (Angiogenin), CXCL12 (SDF-1), and MAST2 (IL-12) are connected using green (text mining), pink (experimentally determined), blue (from curated databases), and grey (co-expression) lines.

Text mining: these biomarkers are identified as having a significant protein interaction group in the abstracts of scientific literature within the String database.

Experimentally determined: these biomarkers are identified as having a protein-protein interaction, determined experimentally, within the String data sets.

From curated databases: these biomarkers are identified as having a link via the String curated database.

Co-expression: these biomarkers are simultaneously expressed, within homo sapiens, in response to a stimulus.

CASP1 (Caspase 1): processes the precursor forms of IL-1β

IL-3: Interleukin-3

TNF (Tumour Necrosis Factor): also referred to as TNF- α

VEGFA (Vascular Endothelial Growth Factor A): belongs to the VEGF family

CCL2(CC motif Chemokine Ligand 2): also referred to as Monocyte Chemoattractant Protein 1 (MCP1)

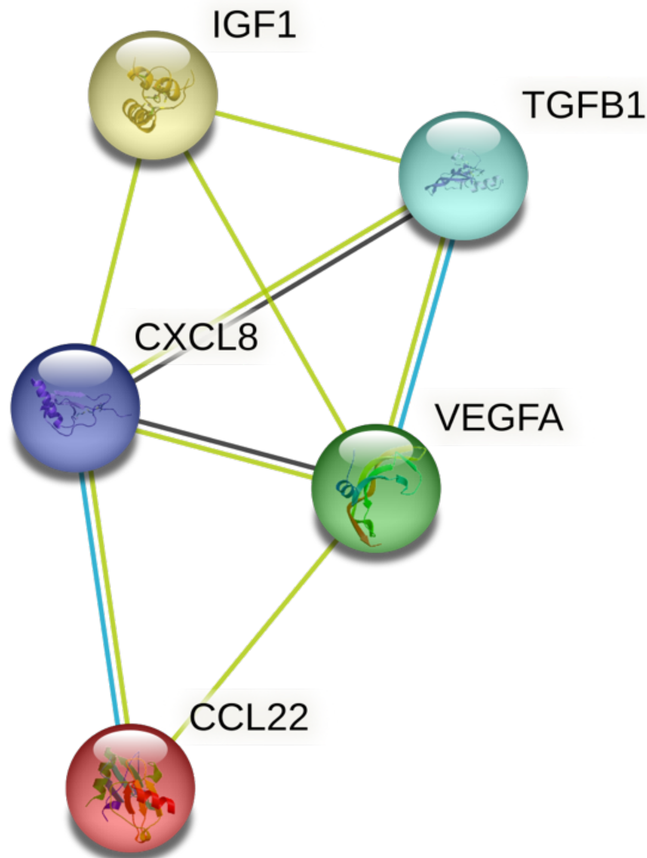
EGF: Epidermal Growth Factor

ANG: Angiogenin

CXCL12 (CXC motif Chemokine Ligand 12): also referred to as Stromal Cell-derived Factor 1 (SDF-1)

MAST2 (Microtubule Associated Serine/Threonine Kinase 2): involved in the regulation of interleukin-12 (IL-12) production

Figure 5.6 Physiological Pathways and Connections between Cytokines and Growth Factors that Responded to Prolonged and Interrupted Sitting Sessions



Connections between five cytokines and growth factors with observed changes from prolonged and interrupted sitting conditions are displayed using a String Diagram. IGF-1, TGF-B1 (TGF-β1), CXCL8 (IL-8), VEGFA, and CCL22 (MDC) are connected using green (text mining), blue (from curated databases), and grey (co-expression) lines.

Text mining: these biomarkers are identified as having a significant protein interaction group in the abstracts of scientific literature within the String database.

From curated databases: these biomarkers are identified as having a link via the String curated database.

Co-expression: these biomarkers are simultaneously expressed, within homo sapiens, in response to a stimulus.

IGF-1: Insulin-like Growth Factor 1

TGF-B1 (TGF- β1): Transforming Growth Factor Beta 1

CXCL8 (CXC motif Chemokine Ligand 8): also referred to as IL-8

VEGFA (Vascular Endothelial Growth Factor A): belongs to the VEGF family

CCL22 (CC motif Chemokine Ligand 22): also referred to as Macrophage-Derived Chemokine (MDC)

6. Discussion

Included in the following chapter is a review of the hypotheses outlined above and the findings of this study, comments on potential explanation of the findings, comments on the strengths and limitations of this study, and recommendations for future directions of research within this topic.

6.1. Hypothesis and Findings

We aimed to determine if four hours of prolonged sitting would increase concentrations of pro-inflammatory cytokines in saliva, would impact salivary vascular and growth factor concentrations, and if there were sex-dependent differences in biomarker response. We also sought to determine if interrupting four hours of sitting with three minutes of moderate intensity walking every 27 minutes would result in a change of concentration of biomarkers compared to prolonged sitting, and if sex mediated the response to sedentary interruptions. We found that prolonged sitting does increase levels of some pro-inflammatory biomarkers in saliva, yet the role of some cytokines as myokines may influence the response to exercise, that certain vascular and growth factors are responsive to periods of prolonged and interrupted sitting, and that sex does play a role in the response of biomarkers to the two conditions. Of the 42 targets of the antibody array kit, there were 26 detectable biomarkers. Summarized in Tables 6.1-6.4 are the detectable biomarkers in response to prolonged and interrupted sitting, with an interpretation of the findings as it relates to the general trends of our hypotheses.

Table 6.1 Cytokines that responded as hypothesized

We hypothesized that most pro-inflammatory cytokines would 1) increase in response to prolonged sitting, 2) decrease in response to interrupted sitting, and 3) that males would be more responsive to prolonged and interrupted sitting				
Cytokine (Chemokine or Myokine	**Increased in Response to Prolonged Sitting**	**Decreased in Response to Interrupted Sitting**	**Sex Differences**	**Interpretation of Findings**
MCP-1	✓ (M/F)		✓	Among both males and females, MCP-1 was higher from baseline to the end of the PS protocol. MCP-1 was higher among females compared to males. A study with a prolonged sitting and breaking prolonged sitting trial found an increase in MCP-1 mRNA in both trials [1].
TNF-α	✓ (F)	✓ (F)	✓	Among females, TNF-α was higher from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. TNF-α was not detected in males in either protocol. Previous research has associated higher TNF-α with sedentary time [2-4]. Some research has shown TNF-α to increase following acute exercise [5] while others state that the anti-inflammatory effects of exercise training leads to reduced concentrations of TNF-α [6]. TNF-α is a myokine, and so has been shown to be responsive to muscular contraction [7, 8]. Current research is contradictory, with some studies describing no

				difference between males and females and some highlighting sex differences [9, 10].
MCSF	✓ (M)		✓	Among males, MCSF was higher from baseline to the end of the PS protocol. No research available, to our knowledge, regarding the response of MCSF to prolonged sedentary time.
MDC	✓ (F)	✓ (F)	✓	Among females, MDC was higher from baseline to the end of the PS protocol and was lower from baseline to the end of the IS protocol. No research available, to our knowledge, regarding the response of MDC in response to prolonged and interrupted bouts of sitting.
MIG	✓ (M)		✓	Among males, MIG was higher from baseline to the end of the PS protocol and was not detected in the IS protocol. No research available, to our knowledge, regarding the response of MIG to prolonged sedentary time.
Oncostatin M	✓ (M)		✓	Among males, Oncostatin M was higher from baseline to the end of the PS protocol. Among females, Oncostatin M was not detected in the PS protocol. No research available, to our knowledge, regarding the response of Oncostatin M to prolonged sedentary time.
IL-8	✓ (F)	✓ (F)	✓	Among females, IL-8 was higher from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. Research has

				documented IL-8 concentrations to increase in response to prolonged sitting and to have an attenuation or abolishment of this increase with sedentary interruptions, which may be dependent on exercise intensity [11-13]. One study, also commented that males may be more responsive to exercise interruptions than females [13].
GRO	✓ (M)		✓	Among males, GRO was higher from baseline to the end of the PS protocol. GRO was not detected in females in either protocol. In a study investigating individuals with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and sedentary controls, GRO concentrations decreased after exercise in both groups [14].
GRO-α	✓ (M)	✓ (M)	✓	Among males, GRO-α was higher from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. GRO-α was not detected in females in either protocol. In a study investigating young sedentary, young active, old sedentary, and old active individuals, GRO-α concentrations were higher among older individuals independent of physical activity group and among young individuals GRO-α concentrations were higher in the active group compared

				to the sedentary group, though not significantly [15].
IL-12 p40/p70	✓ (M)		✓	Among males, IL-12 was minimally higher from baseline to the end of the PS protocol. IL-12 was not detected in females in either condition. Baseline concentrations of IL-12 were slightly higher among young sedentary individuals compared to young active individuals [15].
IL-13	✓ (F)		✓	Among females, IL-13 was higher from baseline to the end of the PS protocol. IL-13 was not detected in the IS protocol in females or either protocol in males. Baseline concentrations of IL-13 were higher among sedentary individuals (young and old) compared to active individuals, though potentially not statistically significant. IL-13 concentrations decreased following acute exercise across all groups [15].
IL-15	✓ (M)		✓	Among males, IL-15 was higher from baseline to the end of the PS protocol. IL-15 was not detected in females in the PS protocol. Baseline concentrations of IL-15 were minimally higher among sedentary individuals (young and old) compared to active individuals, though potentially not statistically significant [15].
Legend: Males = M, Females = F, and Both Sexes = M/F				

Table 6.2 Cytokines that did not respond as hypothesized

We hypothesized that most pro-inflammatory cytokines would 1) increase in response to prolonged sitting, 2) decrease in response to interrupted sitting, and 3) that males would be more responsive to prolonged and interrupted sitting				
Cytokine (Chemokine or Myokine	**Decreased in Response to Prolonged Sitting**	**Increased in Response to Interrupted Sitting**	**Sex Differences**	**Interpretation of Findings**
MCP-1		✓ (F)	✓	Among females, MCP-1 showed a higher pixel density at the end of the IS protocol than at the end of the PS protocol. MCP-1 was not detected in males in the IS protocol. Previous research has documented MCP-1 increasing in response to acute exercise [16] [17].
IL-3	✓ (M)	✓ (M)	✓	Among males, IL-3 was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. IL-3 was not detected in females in either protocol. Limited research is available regarding IL-3 in response to prolonged sitting or exercise, though one study showed that those who are physically active have lower IL-3 concentrations at baseline compared to those that are sedentary [15].

IL-4		✓ (M)	✓	IL-4 was not detected among females in either protocol, or among males in the PS protocol. IL-4 was higher from baseline to the end of the IS protocol among males. Though Pedersen et al., state that exercise may induce a pro-inflammatory cytokine release, followed by a sequence of anti-inflammatory cytokines, such as IL-4 [18], most studies investigating IL-4 in response to exercise show no change in IL-4 concentration [19-21].
MCSF		✓ (M/F)	✓	MCSF was higher from baseline to the end of the IS protocol among males and females. Among males, MCSF showed a higher pixel density at the end of the IS protocol compared to the end of the PS protocol. Among females, MCSF was not detected in the PS protocol. MCSF was higher among males compared to females. Some research has shown that MCSF concentrations increase after exercise [22, 23].
ENA-78		✓ (M/F)	✓	Among males and females, ENA-78 was higher from baseline to

				the end of the IS protocol and not detected in the PS protocol. ENA-78 was higher among males compared to females. One study documented ENA-78, as being higher post-acute exercise and after an eight-week training intervention [24].
MDC	✓ (M)	✓ (M)	✓	Among males, MDC was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. Though there is limited research regarding MDC in response to exercise, one study comments on the beneficial effect of exercise on macrophages [25].
MIG		✓ (F)	✓	Among females, MIG was higher from baseline to the end of the IS protocol and was not detected in the PS protocol. A previous study, investigating women, stated that there was no change in MIG after acute exercise [16].
Oncostatin M		✓ (M/F)	✓	Among males and females, Oncostatin M was higher from baseline to the end of the IS protocol. Among males, Oncostatin M was higher at the end of the

				IS protocol than at the end of the PS protocol. Some animal based research has documented an increase in Oncostatin M concentrations following exercise [26, 27]
IL-8	✓ (M)	✓ (M)	✓	Among males, IL-8 was lower from baseline to the end of the PS protocol and was higher from baseline to the end of the IS protocol. Some studies have documented IL-8 concentrations to increase post-exercise [28], while others have documented no change in IL-8 in response to exercise [15, 29].
Thrombopoietin		✓ (F)	✓	Thrombopoietin was not detected among males in either protocol or among females in the PS protocol. Among females, Thrombopoietin was higher from baseline to the end of the IS protocol. One study stated that Thrombopoietin concentrations were unchanged following high-intensity acute exercise [30].
GRO		✓ (M)	✓	Among males, GRO was higher from baseline to the end of the IS protocol. GRO was higher at the end

				of the IS protocol than at the end of the PS protocol. In a sample of sedentary females, GRO concentrations increased post-acute exercise [16]. In a study investigating young sedentary, young active, old sedentary, and old active individuals, GRO concentrations increased across all groups after acute exercise [15].
RANTES	✓ (M)	✓ (M)	✓	Among males, RANTES was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. RANTES was not detected in females in either protocol. Some studies have documented the decrease of RANTES concentrations post-acute exercise [15, 16], whereas another study documented RANTES to increase following 8 months of training [31].
IL-12 p40/p70		✓ (M)	✓	Among males, IL-12 was higher from baseline to the end of the IS protocol. IL-12 was higher at the end of the IS protocol than at the end of the PS protocol. One study documented that IL-12 p40 increased after

				sub-maximal exercise in a group of sedentary controls [14], while another study noted no change in IL-12 post-exercise [15].
IL-15		✓ (M/F)	✓	Among males and females, IL-15 was higher from baseline to the end of the IS protocol. IL-15 was higher in males compared to females at the end of the IS protocol. IL-15 was higher at the end of the IS protocol than at the end of the PS protocol in males. One study documented an increase in IL-15 after acute exercise among active individuals [15], and another study documented the increase of IL-15 after resistance exercise [32].
IL-1β	✓ (M)	✓ (M/F)	✓	Among males, IL-1β was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. Among females, IL-1β was not detected in the PS protocol and was higher from baseline to the end of the IS protocol. At the end of the IS protocol, IL-1β was higher in males than in females. One study documented an increase of IL-1β after

				acute exercise in physically active individuals [15], and another study documented the increase of IL-1β during and after a 3-hour exercise protocol [33].
Cytokines that were not detected: IL-2, MCP-2, TNF-β, MCP-3, IL-5, IL-6, IL-7, GM-CSF, MIP-1δ, IL-10, I-309, Leptin, IL-1α, TARC, and IFN-γ. This could be due to insufficient protocol duration and/or intensity, the participants being healthy, young, and active individuals, or not being present and/or detectable within saliva.				
Legend: Males = M, Females = F, and Both Sexes = M/F				

Table 6.3 Growth, endothelial, and vascular factors that responded as hypothesized

We hypothesized that most pro-inflammatory vascular/endothelial and growth factors would 1) increase in response to prolonged sitting, 2) decrease in response to interrupted sitting, and 3) have minimal sex differences, or that males would be more responsive.				
Vascular/Endothelial or Growth Factor	**Increase in Response to Prolonged Sitting**	**Decrease in Response to Interrupted Sitting**	**Sex Differences**	**Interpretation of Findings**
EGF	✓ (M)	✓ (M)	✓	Among males, EGF was higher from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. Current research states that EGF concentrations will increase with prolonged sedentary time [17, 34, 35].
IGF-1	✓ (M)	✓ (M)	✓	Among males, IGF-1 was higher from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. One study states that sedentary behaviour could lead to activation of IGF-1 [34], while another study states that exercise was associated with an increase of IGF [36].

Angiogenin	✓ (M/F)	✓ (M/F)	✓	Among males and females, Angiogenin was higher from baseline to the end of the PS and IS protocols. A more profound response to both protocols was observed in males. Current research states that Angiogenin concentrations decrease with prolonged sitting [37, 38], increase with interrupted sitting [39, 40], and that there are potentially no sex differences or a less profound effect among females [37, 40].
VEGF	✓ (M)		✓	Among males, VEGF was higher from baseline to the end of the PS protocol. Current research indicates that VEGF concentrations do not have significant changes with prolonged sitting [41, 42].
SCF	✓ (M)		✓	Among males, SCF was higher from baseline to the end of the PS protocol. SCF was not detected in females in either protocol. No

				research available, to our knowledge, regarding the response of SCF to prolonged sitting.
PDGF-BB	✓ (M)		✓	Among males, PDGF-BB was higher from baseline to the end of the PS protocol. PDGF-BB was not detected in females in either protocol. One study investigating PDGF-BB in young sedentary, young active, old sedentary, and old active individuals states that PDGF-BB concentrations are higher among older individuals regardless of physical activity status [15].
TGF-β1	✓ (M/F)	✓ (M/F)	✓	Among males and females, TGF-β1 was higher from baseline to the end of the PS and IS protocols. Among females, TGF-β1 was higher to a lesser extent in the IS protocol. Among males, TGF-β1 was higher to a greater extent in the IS protocol. Females had overall higher values of TGF-β1 than males. One study states that

				TGF-β1 levels were lower in recreational cyclists compared to sedentary controls [43]. Another study states that after strenuous exercise serum concentrations of TGF-β1 increase [44].
Legend: Males = M, Females = F, and Both Sexes = M/F				

Table 6.4 Growth, endothelial, and vascular factors that did not respond as hypothesized

We hypothesized that most pro-inflammatory vascular/endothelial and growth factors would 1) increase in response to prolonged sitting, 2) decrease in response to interrupted sitting, and 3) have minimal sex differences, or that males would be more responsive.				
Vascular/Endothelial or Growth Factor	**Decrease in Response to Prolonged Sitting**	**Increase in Response to Interrupted Sitting**	**Sex Differences**	**Interpretation of Findings**
EGF	✓ (F)	✓ (F)	✓	Among females, EGF was lower from baseline to the end of the PS and IS protocols, but to a lesser extent at the end of the IS protocol. Some studies indicated that EGF concentrations will decrease in response to exercise [17, 45]. Other studies indicated that EGF would show minimal sex differences or be higher among males [46, 47].
IGF-1	✓(F)	✓(F)	✓	Among females, IGF-1 was lower from baseline to the end of the PS and IS protocols, but to a greater extent at the end of the IS protocol. Some studies state that there was no change in IGF-1 with training [48, 49], while others

				say there is an increase [50, 51], and others say there could be a decrease [52].
VEGF	✓ (F)	✓ (M/F)	✓	Among females, VEGF was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. Among males, VEGF was higher from baseline to the end of the IS protocol. VEGF was higher at the end of the IS protocol than at the end of the PS protocol in males. Current research indicated that sedentary interruptions would increase concentrations of VEGF [36, 53-55], and that VEGF would have no significant differences between males and females [56].
SCF		✓ (M)	✓	Among males, SCF was higher from baseline to the end of the IS protocol. SCF was higher at the end of the IS protocol than at the end of the PS protocol. No research

				available, to our knowledge, regarding the response of SCF to exercise.
PDGF-BB		✓ (M)	✓	Among males, PDGF-BB was higher from baseline to the end of the IS protocol. PDGF-BB was higher at the end of the IS protocol than at the end of the PS protocol. Current research states that PDGF-BB concentrations increase post exercise [44, 57].
SDF-1	✓ (M)	✓ (M)	✓	Among males, SDF-1 was lower from baseline to the end of the PS protocol and higher from baseline to the end of the IS protocol. SDF-1 was not detected in females in either protocol. One study states that aerobic exercise upregulates SCF-1 expression [58].
Growth/Vascular/Endothelial Factors that were not detected: GCSF. This could be due to insufficient protocol duration and/or intensity, the participants being healthy, young, and active individuals, or not being present and/or detectable within saliva.				
Legend: Males = M, Females = F, and Both Sexes = M/F				

6.2. Insights from the Data

6.2.1. Acute prolonged sitting studies

The field of sedentary physiology is growing, with an increasing number of studies focusing on the acute effects of prolonged sitting. However, previously the field has been dominated by bed rest or population-based studies. Each study design has both benefits and limitations and provide different insight into the field of research. Bed rest studies have been the primary way to investigate sedentary physiology in the laboratory setting. These studies require participants to remain bed-ridden for hours to months in various positions. However, these studies lack the replication of sedentary behaviour in the daily life of an average person. Population-based studies primarily use self-report or device-based measurements for sedentary time/behaviour and then investigate the influence of higher volumes of sedentary time over a prolonged period (days to years). However, these studies risk reporting errors, using self-report or device-based measures of sedentary time, and fail to investigate the acute effects of prolonged periods of sitting. Bed rest studies provide a highly controlled environment, while population-based studies have larger samples sizes, however, protocols similar to ours, provide a more representative setting to replicate the patterns of sedentary time in daily living.

Cost is of course a consideration in any study. Compared to bed rest studies which require specialized equipment and a large laboratory setting, acute studies can use a standard chair setup, require less laboratory space, and analysis can be tailored to funding. Both types of work require a prolonged time commitment from the participant, as opposed to the population-based or cross-sectional study design. This time commitment from the participant can lead to difficulties in recruitment. In our study, female participants presented a challenge to recruit. Females have been traditionally underrepresented in the literature, and our recruitment strategies proved difficult to attract females compared to males. We were still able to reach the ideal sample size and achieve a balanced sample (n = 24; 50% female), however, during the final two months of data collection we had to restrict participation to only females. We also offered a percentage incentive, using the established Kinesiology Participant Pool procedure in the Faculty of Health Sciences, Kinesiology. Further investigation may be required to better understand male and female recruitment strategies and improve recruitment methods in the future. Ultimately, by

achieving a balanced sample, it enabled us to investigate sex-differences by pooling the samples into male and female sub-groups.

Acute bouts of sitting are a common part of daily living and can be replicated using an acute sitting protocol. Our exploratory research indicates that a single bout of 4-hours of sitting may lead to a more pro-inflammatory biomarker profile, using a cytokine microarray kit; and that interrupting prolonged sitting with moderate intensity exercise, leads to a change among several biomarkers of interest. Several studies and previous work from our laboratory support the pro-inflammatory nature of sitting, although, the clinically significant increase or decrease in biomarker concentration and specific intensity required to elicit change are unclear. Previous research from our lab found that moderate intensity walking led to an attenuation of the increase of IL-8 [13], while high intensity cycling led to an abolishment of the increase [11]. Future research may need to investigate the influence of sedentary time accumulated from repeated bouts of sitting to replicate daily living and the specific increase or decrease of biomarkers in response to varying levels of exercise intensity.

6.2.2. Saliva as a methodological consideration

Saliva collection is an easy, non-invasive technique to obtain an analyte for sedentary physiology and exercise studies. However, it does present certain challenges compared to blood.

Saliva samples could be contaminated by food, liquid, caffeine, alcohol, oral infections, and can vary depending on behaviour, including smoking, brushing technique, and using mouth wash, etc. Table 3.1, outlines the benefits and limitations of saliva use and a review by Szabo and Slavish, outlines methodological considerations when using saliva to measure biomarkers [59]. Compared to saliva, blood has been well-documented in its use of measuring biomarkers. However, it does present some unique challenges, including acute stress produced during invasive collection and more rigorous sample preparation and storage. Using saliva, may have contributed to the recruitment of sufficient participants because collection was less invasive compared to a study in which capillary or venous blood samples would be required. Our study provides insight into the future directions of saliva as a methodological option, as discussed by Szabo and Slavish. We

attempted to control for potentially confounding behaviours to investigate how a wide array of biomarkers respond to acute bouts of prolonged and interrupted sitting. Specifically, in this study, the volume of saliva collected per participant per condition varied from approximately 1.5-3.0 mL. This was a relatively small amount of saliva, and the volume collected depended heavily on the participant following the pre-participation instructions to drink 1.0 L of water on the morning of participation. Whether the participant had consumed the desired amount of water was apparent during pre-protocol saliva collection with some participants easily providing 3.0 mL or more, while others struggled to provide 1.5 mL. This then became challenging during the post-protocol collection, as most individuals refrained from drinking excessive amounts of water during the sitting protocols to avoid using the restroom. A future consideration could be for a standardized amount of water to be consumed throughout the protocol and to implement pre-determined restroom breaks. For example, Peddie et al., had participants walk the required distance to the restroom location at specified times, whether or not they needed to actually use the restroom [60]. This would then standardize the protocol, avoid difficulties collecting sufficient saliva, and avoid potential discomfort for the participant. Additionally, oral health may have influenced salivary biomarker concentrations, though this study excluded participants who had undergone recent dental procedures and who smoked, oral health, such as gingivitis or acute cuts/sores in the mouth could have increased local inflammation, therefore potentially confounding salivary biomarker concentrations [61-63]. Though this would be difficult to control for in an experimental study of this nature, future considerations could include a visual check of the oral cavity prior to participation, standardization of a soft-bristle toothbrush in the days leading up to participation to avoid abrasion of the gums, and an oral hygiene questionnaire as mentioned by Bhattarai et al. [61]. The method of collection, using an oral swab (Salimetrics SalivaBio, Salimetrics LLC, State College, PA, USA), which was then centrifuged in the original container, made saliva collection easy for both the researcher and participant.

6.2.3. Bioenergetic Considerations

A potential limitation was the standardized breakfast provided and its potential impact on results. The nutritional values of the standardized meals we provided (590 Calories; Fats 6.5g, Carbohydrates 127g, Proteins 11.1g) included breakfast and a snack and had a

relatively high carbohydrate value. Carbohydrate status has been documented to influence biomarker levels, specifically myokines [64, 65]. This then may have led to an influence of biomarker concentration, as this was an exploratory study, investigating the response of novel biomarkers to prolonged and interrupted bouts of sitting. Providing the standardized meal in both conditions attempted to control for the potential influence of carbohydrate status, however, future research may look to investigate the role of nutrition on specific biomarker response. Additionally, diet and sleep in the days leading up to participation may have influenced salivary biomarker concentrations. Dietary patterns have been associated with variations in the saliva [66]. Future research should investigate the role of different dietary patterns on biomarker responses to sitting and exercise, as well as acute changes to dietary patterns leading up to study participation; however, acute standardization of diet may not be sufficient to elicit a change in salivary profiles as stated by Walsh et al.; instead studies may need to consider recent dietary intake and timing of sample collection to reduce potential confounders [67]. Sleep may also influence salivary biomarker concentrations. Poor sleep quality and sleep disorders have been associated with altered levels of salivary stress hormones and inflammatory cytokines [68]. Alterations in sleep have been associated with adverse metabolic effects and an increase in pro-inflammatory biomarkers and some cytokines have been documented as following a sleep-wake or circadian rhythm cycle [69, 70]. Specifically, IL-6 and TNF-α have been documented as having higher concentrations in those that suffer from sleep alterations [68, 71, 72]. Comments from the participants regarding the food, included that it was a large quantity of oatmeal to consume first thing in the morning, and that the food (oatmeal, juice box, and granola bar) was relatively bland. Future studies may look at providing a smaller initial meal and then a larger secondary meal. This does present some challenges in terms of food storage, expiration, and cost; since larger and more flavourful items may be more difficult to store, require refrigeration, have a shorter shelf-life, and therefore be more expensive. However, there are some options to incorporate more variety while maintaining the non-perishable nature and cost-efficient considerations, such as fruit cups, instant noodles, or canned goods. These could be heated/prepared in the laboratory setting using similar methods to the oatmeal preparation, while providing the participant with a potentially more desirable option. Providing a smaller initial meal and then a larger

secondary meal may be more desirable to participants as they indicated that they regularly only had a small meal for breakfast and then consumed more food around mid-day. However, this may influence biomarker response due to the influence of carbohydrates and oral contamination which would need to be considered in future studies.

Another consideration is exercise intensity. A previous pilot study from our laboratory investigated IL-8 in response to prolonged and interrupted sitting with the interruptions occurring at the end of each hour and using a maximal intensity cycling intervention, likely providing a greater exercise stimulus due to intensity. This resulted in the response of IL-8 being abolished within the sedentary interruption protocol [11]. However, another study from our laboratory of the same protocol as this thesis, investigated the response of IL-8 to prolonged and interrupted sitting, with the interruptions occurring at the end of every half-hour and using a moderate-intensity walking protocol, likely providing a smaller exercise stimulus. This resulted in the response of IL-8 being attenuated within the sedentary interruption protocol, but with males being more responsive [13]. Within our study, we observed that exercise intensity may play a role in the response of certain biomarkers and the interplay of sex and intensity may also influence biomarker response.

6.2.4. Cytokine signature

Another consideration in our study, is cytokine signature, the concept that cytokine actions are specific based on target and stimulus, and is context dependent on a condition or state. This is clearly apparent in the response of IL-8, among males within our study as it decreased from baseline to the end of the 4-hour prolonged sitting protocol and increased from baseline to the end of the 4-hour interrupted sitting protocol. This is contrary to previous research from our laboratory, which has documented IL-8 as increasing with prolonged sitting and decreasing with sedentary interruption [13], but is in alignment with previous research that has found an increase in concentrations of IL-8 mRNA and protein expression in the skeletal muscle in response to exercise [28, 73]. This variation in response could then be attributed to IL-8 acting as a myokine in this setting, as myokines have been shown to respond to muscular contractions [7, 8]. Relationships between responsive and detectable cytokines and growth factors were assessed using the String Diagram generator [74]. In Figure 5.6, relationships between five biomarkers that responded to prolonged and

interrupted sitting are displayed, this includes IL-8. Potential sex-differences between biomarkers are displayed in Figure 5.5. Though we are not proposing that this is the definite source of IL-8 or any biomarker within our study, it is important to consider during the interpretation of results. However, as cautioned by Szabo and Slavish, it is difficult to make claims about system-wide changes based on salivary biomarkers, though it can be inferred. There are many factors that could influence salivary biomarkers, and the actual source of salivary biomarkers is debatable. Therefore, without confirmation of its release source, which requires more invasive sampling, such as muscle biopsies, the cytokine source and target cannot be determined. Future work should consider working with experts to determine saliva as a clinical analyte, the investigation into cytokine sources, the influence of sex on cytokine response, and more exploratory work, like ours, is needed to identify what is present and responsive in saliva.

6.3. Limitations

Limitations of this research includes the use of saliva without blood samples for comparison. As mentioned previously, the variation of collected saliva volume, due to some participants consuming sufficient water prior to collection and some not, sometimes made saliva collection difficult and may have confounded results, to an unknown extent. However, saliva collection was much easier compared to a capillary prick blood collection method used in a previous study in this lab [13] and provided sufficient protein to complete analysis. Though potentially not a limitation due to the lack of research of prolonged sitting on young, healthy, individuals, there has been documentation of the inflammatory response differing with age and health status, which makes our research difficult to generalize to a larger population. The timing of data collection for the study (October 2021-February 2022) and period of the semester for our sample population of university students, may have influenced salivary biomarker concentrations due to psychological stress or seasonality [75]. Due to the style of analysis, using pooled samples and an antibody membrane array with average pixel density as the output, there is no unit to quantify our data, making statistical analysis essentially impossible. Instead, our analysis relied on the average pixel density counts and graphical representation of the biomarkers detected on the membrane. This provides a trend among the pooled samples but limits investigation into individual responses and does not provide specific statistics to reference. Using an

ELISA could provide insight into individual responses, however, the limited amount of sample remaining, and the large number of novel biomarkers identified in the exploratory part of this analysis made it difficult to select which biomarker to focus on, while considering time and cost for this study.

6.4. Future Directions

Future research should aim to examine the dose-response relationship of biomarkers to exercise intensity. The influence of sex on biomarker response is apparent in our study, however future work should investigate the response of specific biomarkers on an individual level and the interaction of exercise duration, exercise intensity, and sex on biomarker variations. To build from this study, the next study could include a randomized cross-over with low, moderate, and high exercise intensity interruptions, to try to better understand the influence of intensity on biomarker response. A future study could also implement pre-determined restroom breaks and different standardized meals, while investigating if these changes alter the response of certain biomarkers. Future research could also identify biomarkers of particular interest or that are considered gaps in the literature from this study, particularly growth and endothelial factors, and complete individual level analysis using ELISAs. There is also still more research required on the influence of sex on biomarker response, so future research could aim to be sufficiently powered to complete sex-based analysis using individual level data. Future work should aim to identify other biomarkers present and responsive in saliva and standardize saliva sample collection procedures.

6.5. Conclusions

In conclusion, four hours of prolonged sitting lead changes in salivary biomarker concentrations, with variations dependent on sex and sedentary interruptions with regular, moderate-intensity walking. There appears to be sex differences in biomarker presence at baseline and biomarker response to prolonged sitting and to sedentary interruptions with exercise. Several cytokines and growth and vascular factors were observed to be responsive to prolonged and interrupted bouts of sitting. Future research should aim to understand the underlying mechanisms of biomarker response, the effect of exercise intensity on

biomarker response, and the individual response of biomarkers to prolonged and interrupted bouts of sitting.

6.6. References

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7. Appendices

Appendix A.

A.1. Eligibility Questionnaire

Eligibility Questionnaire

1. Age: ________

2. Sex:

   Female ☐

   Male ☐

If female, are you currently pregnant?

   Yes ☐

   No ☐

3. Do you have any chronic diseases such as asthma, heart disease, irritable bowel syndrome, arthritis, or others?

   a. Yes

   b. No

   If yes, please describe:

4. What is your current:

   1. Height (cm):
   2. Weight (kg):

5. Do you currently have an infection, cold, or flu like symptoms?

   1. Yes
   2. No

   If yes, please describe.

6. Are you currently taking any prescription or over the counter medications regularly?

1. Yes
2. No

If yes, please list the medications here: ________________________________

7. Do you have any injuries that would limit your ability to run or walk on a treadmill? (e.g.

   knee injury)

   1. Yes
   2. No

   If yes, please describe the injury here:

____

8. Have you ever been a regular smoker?

   1. Yes
   2. No

   If yes, please indicate for how long:

____

9. Do you have any dietary restrictions?

   1. Yes
   2. No

   If yes, please indicate what kind: ________________________________

   Please note, we will be providing a standard breakfast and snack that must be consistent across participants, therefore, this information is important for us to determine your eligibility.

10. At the present time do you smoke daily, occasionally, or not at all?

    1. Daily (at least 1 cigarette per day for the last 30 days)
    2. Occasionally (at least 1 cigarette in the last 30 days but not every day)
    3. Not at all (you did not smoke at all in the last 30 days)

11. Do you have a history of dental injuries and/or infections?

    1. Yes
    2. No

If yes, please describe the injury and/or infection and the date at which this occurred: ____

____

____

____

12. Please confirm that you are a student at Ontario Tech University.

    Yes

    No

A.2. Informed Consent

ONTARIO TECH UNIVERSITY

ELIGIBILITY ASSESSMENT CONSENT

Study Title: Physiological responses to prolonged and interrupted sitting

Name of Principal Investigator: Shilpa Dogra, PhD

Research Team: Nicholas O'Rourke, BHSc; Emmeline Meens-Miller, BHSc; Julia Green-Johnson, PhD.

Overview

Thank you for your interest in our research study. There are several sections to this document. First, there is a brief explanation of how we will assess your eligibility, and what we will do with the data we collect through our eligibility questionnaire. Next, you will find an information letter containing several details for the full study. You can read this to determine if you are still interested in participating, or if you have any questions for us. You will also find a COVID-19 section at the end of this document. Please read this carefully to understand the steps we are taking to minimize risks associated with the virus.

Eligibility Questionnaire

In order to determine whether you are eligible for this study, we have a short questionnaire for you to complete. This questionnaire includes personal information such as your health status, dietary restrictions, and lifestyle behaviours. Due to the nature of this data, we want to obtain your consent to collect the data.

Please note, your data will be kept confidential. If you would like, it can be sent to us via email, or, you can come to the laboratory where we can provide a printed copy. Once we have determined whether you are eligible or ineligible for the study, we will destroy this data. In other words, no print or digital copies of this information will be retained.

As a reminder, the security of e-mail messages is not guaranteed. Messages may be forged, forwarded, kept indefinitely, or seen by others using the internet. Thus, if you do return the eligibility questionnaire to us via email, we ask that you password protect the document to improve security. You can communicate this password to us by leaving a message on our laboratory voicemail. Please also be sure to delete the email you sent us.

CONSENT

I understand how the data from my eligibility questionnaire will be handled. Any questions regarding this part of the study have been answered to my satisfaction.

I understand my consent to participate, or to not participate in this study is voluntary. I also understand my right to withdraw from any part or all of this study for any reason. I waive no legal rights by participating in this study.

If I have any questions regarding my rights as a research participant, or about any issues relating to this study, I will contact Dr. Shilpa Dogra at 905.721.8668 ext. 6240 or at Shilpa.Dogra@ontariotechu.ca.

I hereby consent to participate in this study.

__________________________ ____________________ ______/______/________
Participant (Print Name) Signature Date

For a member of the research study: I have ensured the named participant above has thoroughly understood all aspects of this research study, and have answered all questions to their satisfaction.

__________________________ ____________________ ______/______/________
Research Member (Print Name) Signature Date

Participant Concerns and Reporting:

Please read this consent form carefully and feel free to ask the researcher any questions that you might have about the study. If you have any questions about your rights as a participant in this study, complaints, or adverse events, please contact the Research Ethics Office at (905) 721-8668 ext. 3693 or at researchethics@ontariotechu.ca

If you have any questions concerning the research study or experience any discomfort related to the study, please contact the researcher at 905.721.8668 ext. 6240 or shilpa.dogra@ontariotechu.net.

By signing this form you do not give up any of your legal rights against the investigators, sponsor or involved institutions for compensation, nor does this form relieve the investigators, sponsor or involved institutions of their legal and professional responsibilities

This study has been approved by the OTU Research Ethics Board REB#16473.

ONTARIO TECH UNIVERSITY

OntarioTech Health Sciences

Study Title: Physiological responses to prolonged and interrupted sitting

Name of Principal Investigator: Shilpa Dogra, PhD

Research Team: Nicholas O'Rourke, BHSc; Emmeline Meens-Miller, BHSc; Julia Green-Johnson, PhD.

Study Information

Introduction:
You are invited to participate in a research study that is being conducted at Ontario Tech University (OTU). Throughout this document you will find the study purpose, procedure, benefits and risks, as well as your right to refuse to participate or withdraw from the study. Please thoroughly read and understand all sections of this document before you agree to participate in this study. This is known as the informed consent process. Should you have any questions concerning any of the information, words, or your rights, please contact the researchers above to gain full understanding before signing this consent form.

Purpose & Explanation of the Study:
The purpose of the proposed study is to compare markers of inflammation between a continuous sitting session and a session of sitting interrupted by short periods of walking. To do so, we will compare your response to 4 hours of continuous sitting with your response to 4 hours of sitting interrupted by 3 minutes of walking every half hour.

Eligibility:
In order to be eligible, you must be between the ages of 18-30 years, have a body mass index <30 kg/m^2, must be a non-smoker, and not be taking any medications (such as NSAIDs) that would affect your inflammatory levels, must not be pregnant, and not suffering any acute infections. You must also have no major cardiometabolic, respiratory, or musculoskeletal conditions that would impact your ability to run and walk on a treadmill, and you must be able to consume the standard food provided. You have already completed an eligibility questionnaire.

At the first session, we will also be measuring your resting heart rate, blood pressure, height and weight to confirm eligibility in the study.

Assessment Procedures:
As a participant in this study, you will be asked to attend 3 laboratory sessions. During the first laboratory session we will ask you to provide a saliva sample, perform a maximal exercise test, and then fit you with a small device that measures your daily activity levels. The exercise test will be conducted on a treadmill, starting with a warm-up pace, and progressively getting harder until you reach a point where you can no longer exercise; this will be followed by a cool-down. The exercise portion usually lasts between 8-12 minutes. We will then fit you with a small device to be worn for a seven-day period. The goal is to always keep the device on, including during

sleep and any water-based activities (shower, swimming etc.) The device will be removed the following week at the beginning of your second session.

For each laboratory session, you will arrive at the laboratory fasted, having had at least 1L of water that morning. We also ask that you refrain from alcohol and intense exercise at least 24 hours prior to the session. We will also confirm that no new infections or conditions have arisen between sessions and that no new medications (that impact inflammation) have been taken. We also ask that you be prepared to exercise, wearing the appropriate clothing (athletic clothing and shoes) for comfort and ease of data collection. We also recommend you bring a device, such as a laptop, to keep yourself busy during the sessions. Before beginning the experiment, we will guide you through the process to obtain your own blood via a finger-prick method, we will also collect a saliva sample, measure your resting heart rate and blood pressure, provide you with breakfast, and fit a small strain gauge on your leg. The 4-hour experimental protocol will then begin. This will either be a) 4 hours of continuous sitting or b) 4 hours of sitting interrupted by 3 minutes of walking every 30 minutes. During the 4-hour experimental procedure, you will be allowed to complete an activity of your choosing so long as you are seated (e.g. reading, computer work, watching a movie, etc.). At the midway point of the experimental condition, you will be provided with a granola bar and juice as a snack. Following the experimental procedure, a finger prick blood sample and saliva sample will again be collected. Each session should be completed by approximately 2:30 pm.

Participant Compensation:
You will not be paid for your participation in this study; however, you have much to gain! You will be sent your personal results in the form of an email at the end of the study. If you are a Kinesiology student, you are also able to obtain bonus marks in your Kinesiology courses this semester. The bonus marks will be distributed as per your selection in the Participant Pool survey sent to you at the beginning of the semester. There are 4 components to this study: Maximal exercise test session (1%), activity monitor data (2%), each of the two sitting sessions (1.5% each). If you withdraw from the study, you will receive the bonus marks for the sessions commenced.

Risks and Participant Safety:
Participation in any research study is associated with some risks. The potential risks of this study include feelings of shortness of breath, quickened heart rate, light headedness, and muscular discomfort during and following exercise, and feeling coerced in to participating in the study. You may also experience feelings of stiffness or soreness due to sitting for an extended period. Additionally, you may feel discomfort at the site of the fingertip prick, and have some skin discomfort from wearing the activity monitor. To minimize these risks and to ensure your safety throughout this study, the researchers involved with the study are highly trained and supervised by an Certified Exercise Physiologists, have current CPR training. Additionally, an emergency action plan is posted in the laboratory where sessions are taking place. We will also encourage you to follow all instructions closely, and immediately report any unusual exercise related symptoms.

COVID-19 Risks and Safety

At the end of this document, you will find a detailed description of how we will work to minimize the risks associated with COVID-19. We encourage you to contact us if you have any questions or concerns with regards to COVID-19 safety.

Benefits:
There are numerous benefits to you as a participant in this study. Engaging in the study will provide you with information related to your fitness and cardiovascular health. Participating in this study will also allow you to learn about the effects of interrupting extended sitting time on your health.

Cost of Participating:
There are no costs associated with participation in this study. There will be no reimbursement for any costs incurred for participating in this study (e.g. transportation fees etc.).

Withdrawal:
You have the right to withdraw from the study without any consequence and will be allowed to do so at any point during the study. If you would like to withdraw from the study, please contact the researcher via email at nicholas.orourke@ontariotechu.net or in person. In addition, any data collected from you can be withdrawn and destroyed. Please notify us if you would like your data to be destroyed. You have the right to withdraw your data at any point during the study or for up to 4 weeks following completion of your participation. A reminder, if you withdraw from the study, you will receive the bonus marks for the sessions commenced.

Participant Confidentiality:
At each session, the PI and an assistant will be present to collect data. Following the session, only the research team will have access to your data. Your data will be kept confidential and will be coded (therefore stored anonymously). All hard copies of your data will be stored in a locked cabinet in a laboratory at OTU. Furthermore, identifier codes will be stored in a separate office. The data will be saved on an OTU Google Drive. Your data may be used for secondary use if it is requested for use in a systematic review. In this case, identifier codes would not be provided, to ensure your confidentiality.

Conflict of Interest
You may be a student in the PIs courses or the TA's lab sections. Please note, there are no additional benefits or penalties associated participation or withdrawal related to this study. We will be following the standard protocols outlined in the Participant Pool.

Results
If you are interested in learning about the results of this study, you can read the thesis of Nicholas O'Rourke. This is expected to be published in our library within the next 12 months. Additional results will be available in the thesis of Emmeline Meens-Miller, 24 months from now.

CONSENT

I understand the procedures, potential risk and benefits of this study. Any questions regarding this study have been answered to my satisfaction.

I understand my consent to participate, or to not participate in this study is voluntary. I also understand my right to withdraw from any part or all of this study for any reason. I waive no legal rights by participating in this study.

If I have any questions regarding my rights as a research participant, or about any issues relating to this study, I will contact Dr. Shilpa Dogra at 905.721.8668 ext. 6240 or at Shilpa.Dogra@ontariotechu.ca.

Please indicate if you consent to your data being used for purposes of secondary analysis.

☐ - Yes
☐ - No
(If no, we will not use your data for future studies.)

I hereby consent to participate in this study.

__________________________ ____________________ ______/______/________
Participant (Print Name) Signature Date

For a member of the research study: I have ensured the named participant above has thoroughly understood all aspects of this research study, and have answered all questions to their satisfaction.

__________________________ ____________________ ______/______/________
Research Member (Print Name) Signature Date

Participant Concerns and Reporting:

Please read this consent form carefully and feel free to ask the researcher any questions that you might have about the study. If you have any questions about your rights as a participant in this study, complaints, or adverse events, please contact the Research Ethics Office at (905) 721-8668 ext. 3693 or at researchethics@ontariotechu.ca

If you have any questions concerning the research study or experience any discomfort related to the study, please contact the researcher at 905.721.8668 ext. 6240 or shilpa.dogra@ontariotechu.net.

By signing this form you do not give up any of your legal rights against the investigators, sponsor or involved institutions for compensation, nor does this form relieve the investigators, sponsor or involved institutions of their legal and professional responsibilities

This study has been approved by the OTU Research Ethics Board REB#16473.

COVID-19 Related Precautions

General Notes

- All researchers involved with this study are vaccinated with both shots of the COVID-19 vaccine, and will be carefully following public health guidelines to minimize their risk of exposure and transmission.
- 24h prior to each session you will be required to complete a COVID-19 self-assessment and indicate to the researcher if you have answered "yes" to any of the questions asked.
- All researchers will wear a mask and face shield/goggles at all times. You will be asked to **bring a mask or face covering from home**. It will be mandatory that you wear your mask or face covering until you enter the laboratory. In the event that you forget your mask or face covering, you will be provided with a surgical mask.

Laboratory Session:

- The research team is comprised of 4 researchers. Only two of these researchers will be present at a time during each session. Both researchers present will wear a face shield/goggles and a face mask at all times.
- You will be asked to arrive on campus dressed in active wear that you are comfortable running in. Please use the washroom prior to arrival; however, please note that a washroom is available if needed.
- One member of the research team will greet you at the ***rear entrance*** of the UA building and ask that you sanitize your hands with a 70% hand sanitizer solution. You will be required to wear your mask before entering the building. If you forget your mask a disposable mask will be provided.
- You will then be escorted to the laboratory via a short corridor. (Note: Access to this corridor is restricted to all personnel except the researchers and participants of this study)
- To maximize your safety, all surfaces and touch points will be sprayed and wiped down with a 70% alcohol solution before you enter the laboratory. Once inside the laboratory you may remove your mask or face covering.
- Once you have completed your laboratory session all contact surfaces will be sprayed and wiped down with a 10% bleach solution.
- Prior to exiting the laboratory, you will be asked to put your mask back on and use the provided hand sanitizer once more.
- A member of the research team will escort you out of the UA building using the same restricted corridor.
- **Expelled air:** During the maximal exercise test you will be exhaling through a tube that expels air into the room. At present, the nature of airborne transmission is unknown under such conditions. As such, both researchers present will always be wearing a face shield/goggles and medical style mask.

Note: Physical distancing cannot be practiced inside the lab due to the nature of the research.

Participant Confidentiality – Contact Tracing

- We will be collecting your name and phone number that we must retain in order to follow up with you and/or conduct contact tracing if you may have been exposed to COVID-19.
- In some cases, this may need to be shared with the University or Public Health, and as a result, we cannot guarantee privacy and confidentiality of your participation in the study.
- Contact information will be kept separate from data collection through the research study to allow for de-identification of the research data.
- Although your data will be kept confidential and stored anonymously, we cannot guarantee anonymity, as the personal contact information does identify you as a participant.
- In the event that you develop any COVID-19 symptoms or test positive after the study or at any point during the study, you must contact the researchers as soon as possible.

This study has been approved by the Ontario Tech University Research Ethics Board (REB) [insert REB # assigned] on [insert date].

Any questions regarding your rights as a participant, complaints or adverse events may be addressed to Research Ethics Board through the Ethics and Compliance Officer - please contact the Research Ethics Office at (905) 721-8668 ext. 3693 or at researchethics@ontariotechu.ca

Face-to-Face/In-Person Research Consent Form Addendum for COVID-19

Title of Research Study: Physiological responses to prolonged and interrupted sitting

Name of Principal Investigator (PI): Shilpa Dogra

PI's contact number(s)/email(s):

Phone: (905)721-8668 ext. 6240

Email: Shilpa.Dogra@ontariotechu.ca

Names Student Leads: Nicholas O'Rourke, BHSc; Emmeline Meens-Miller, BHSc

Please note: At this point in time, the risk of Omicron variant of concern in Ontario is high and the risks of further transmission, severe disease, reinfection, and breakthrough infection in Ontario is moderate with a high degree of uncertainty. The overall risk assessment may change as new evidence emerges (Public Health Ontario, December 2021). We will keep you informed on these changes.

I agree to participate in this study taking place at Ontario Tech University during the current COVID-19 pandemic. I understand that my participation is optional. I confirm that I have read and understood the consent form and have been advised on the potential risks related to in-person face-to-face research involving human participants at this time.

If you feel that you are in a vulnerable group with respect to COVID-19 effects (e.g. senior, immunocompromised, living with individuals that may be susceptible to COVID-19), it may be best that you do not participate in the study.

Because you are coming on campus, the following safety protocols must be followed:

- Screening
- Use of non-medical masks or face covering while participating in the research study
- Follow instructions provided to you with respect to arriving at the study location, including entry points, designated waiting areas and washrooms, timing of arrival
- Hand washing
- Precautions taking public transit or travelling to the research site
- Physical distance (maintaining 2-meter distance from others)
- Personal Protective Equipment (PPE) provided to participants by research team

We will be collecting personal contact information that we must retain in order to follow up with you and/or conduct contact tracing if you may have been exposed to COVID-19 in coming to the research site. As a result, we cannot guarantee privacy and confidentiality of your participation in the study.

We cannot guarantee anonymity, as the personal contact information does identify you as a participant.

Contact information will be kept separate from data collection through the research study to allow for de-identification of the research data.

During this time, the university may request information relating to all people entering and exiting our campus. As such please be advised that it may not be possible to keep your participation in a study confidential; however, no information about the data you share with us in the study will be shared outside of the research team.

You maintain your right to withdraw from the study, including research data. If you do withdraw, we will continue to maintain your contact information and will only give it Durham Public Health and the University if required for contact tracing.

There may be additional risks to participating in this research during the COVID-19 pandemic that are currently unforeseen and, therefore, not listed in this consent form.

If you think you have COVID-19 symptoms or have been in close contact with someone who has it, use the Government of Ontario's COVID-19 self-assessment tool and follow the instructions it provides to seek further care. In addition, you must inform the Principal Investigator immediately for follow up.

By checking each of the boxes below, I acknowledge and agree with the statements as follows:

- ⬜ I have either been fully vaccinated with an approved government vaccine, or I have chosen not to be vaccinated.
- ⬜ I acknowledge and accept that there is a risk that I could be exposed to COVID-19 while participating in this research project, despite the approved precautions and protocols that have been put in place.
- ⬜ I acknowledge and accept that while participating in the study, the researchers may need to be closer than the recommended social distancing guidelines in order to carry out the experimental protocols and/or procedures.
- ⬜ I acknowledge and confirm that I am willing to accept this risk as a condition of attending the university to participate in research.
- ⬜ I acknowledge and understand that there may be unknown risk related to COVID-19.
- ⬜ I confirm that the study team has answered all my questions about the study and has advised me of all the risks related to in-person face-to-face research for this study.
- ⬜ I acknowledge that participating in this study may involve third party risks to others where I may expose individuals that I live with or am in close contact with.

Consent to Participate:

1. I have read the consent form and understand the study being described;
2. I have had an opportunity to ask questions and those questions have been answered. I am free to ask questions about the study in the future;
3. I freely consent to participate in the research study, understanding that I may discontinue participation at any time without penalty. A copy of this consent form has been made available to me.

Print Study Participant's Name	Signature	Date

My signature means that I have explained the study to the participant named above. I have answered all questions.

Print Name of Person Obtaining	Signature	Date

A.3. Get Active Questionnaire

Get Active Questionnaire

CANADIAN SOCIETY FOR EXERCISE PHYSIOLOGY –
PHYSICAL ACTIVITY TRAINING FOR HEALTH (CSEP-PATH®)

Physical activity improves your physical and mental health. Even small amounts of physical activity are good, and more is better.

For almost everyone, the benefits of physical activity far outweigh any risks. For some individuals, specific advice from a Qualified Exercise Professional (QEP – has post-secondary education in exercise sciences and an advanced certification in the area – see csep.ca/certifications) or health care provider is advisable. **This questionnaire is intended for all ages – to help move you along the path to becoming more physically active.**

☐ I am completing this questionnaire for myself.

☐ I am completing this questionnaire for my child/dependent as parent/guardian.

PREPARE TO BECOME MORE ACTIVE

The following questions will help to ensure that you have a safe physical activity experience. Please answer **YES** or **NO** to each question before you become more physically active. If you are unsure about any question, answer **YES**.

YES	NO	
		1 Have you experienced **ANY** of the following (A to F) **within the past six months**?
○	○	**A** A diagnosis of/treatment for heart disease or stroke, or pain/discomfort/pressure in your chest during activities of daily living or during physical activity?
○	○	**B** A diagnosis of/treatment for high blood pressure (BP), or a resting BP of 160/90 mmHg or higher?
○	○	**C** Dizziness or lightheadedness during physical activity?
○	○	**D** Shortness of breath at rest?
○	○	**E** Loss of consciousness/fainting for any reason?
○	○	**F** Concussion?
○	○	**2** Do you currently have pain or swelling in any part of your body (such as from an injury, acute flare-up of arthritis, or back pain) that affects your ability to be physically active?
○	○	**3** Has a health care provider told you that you should avoid or modify certain types of physical activity?
○	○	**4** Do you have any other medical or physical condition (such as diabetes, cancer, osteoporosis, asthma, spinal cord injury) that may affect your ability to be physically active?

NO to all questions: go to Page 2 – ASSESS YOUR CURRENT PHYSICAL ACTIVITY

YES to any question: go to Reference Document – ADVICE ON WHAT TO DO IF YOU HAVE A YES RESPONSE

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THE GOLD STANDARD IN EXERCISE SCIENCE AND PERSONAL TRAINING

Get Active Questionnaire

ASSESS YOUR CURRENT PHYSICAL ACTIVITY

Answer the following questions to assess how active you are now.

1 During a typical week, on how many days do you do moderate- to vigorous-intensity aerobic physical activity (such as brisk walking, cycling or jogging)? ☐ DAYS/WEEK

2 On days that you do at least moderate-intensity aerobic physical activity (e.g., brisk walking), for how many minutes do you do this activity? ☐ MINUTES/DAY

For adults, please multiply your average number of days/week by the average number of minutes/day: ☐ MINUTES/WEEK

Canadian Physical Activity Guidelines recommend that adults accumulate at least 150 minutes of moderate- to vigorous-intensity physical activity per week. For children and youth, at least 60 minutes daily is recommended. Strengthening muscles and bones at least two times per week for adults, and three times per week for children and youth, is also recommended (see csep.ca/guidelines).

GENERAL ADVICE FOR BECOMING MORE ACTIVE

Increase your physical activity gradually so that you have a positive experience. Build physical activities that you enjoy into your day (e.g., take a walk with a friend, ride your bike to school or work) and reduce your sedentary behaviour (e.g., prolonged sitting).

If you want to do **vigorous-intensity physical activity** (i.e., physical activity at an intensity that makes it hard to carry on a conversation), and you do not meet minimum physical activity recommendations noted above, consult a Qualified Exercise Professional (QEP) beforehand. This can help ensure that your physical activity is safe and suitable for your circumstances.

Physical activity is also an important part of a healthy pregnancy.

Delay becoming more active if you are not feeling well because of a temporary illness.

DECLARATION

To the best of my knowledge, all of the information I have supplied on this questionnaire is correct.
If my health changes, I will complete this questionnaire again.

I answered NO to all questions on Page 1

Sign and date the Declaration below

I answered YES to any question on Page 1

Check the box below that applies to you:

☐ I have consulted a health care provider or Qualified Exercise Professional (QEP) who has recommended that I become more physically active.

☐ I am comfortable with becoming more physically active on my own without consulting a health care provider or QEP.

Name (+ Name of Parent/Guardian if applicable) [Please print] | Signature (or Signature of Parent/Guardian if applicable) | Date of Birth

Date | Email (optional) | Telephone (optional)

With planning and support you can enjoy the benefits of becoming more physically active. A QEP can help.

☐ Check this box if you would like to consult a QEP about becoming more physically active.
(This completed questionnaire will help the QEP get to know you and understand your needs.)

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A.4. Session 1 Data Collection Sheet

1. **Session 1: Data Collection Sheet**

Participant No.		**Date**	
Participant Arrival Time		**Session to be Completed Today**	1
Age		**Sex**	

☐ Informed Consent
☐ Get Active Questionnaire (*appended at end of doc*)
☐ Participant reminded right to withdraw
☐ Followed instructions

Obtain Emergency Contact information:
Name:
Relationship:
Phone Number:

Saliva Samples
☐ Saliva SOP followed
☐ Eppendorf tubes:
- Number obtained:
- ☐ Labeled

☐ Breakfast provided

Anthropometrics

Height (cm)	
Weight (kg)	

Resting Heart Rate & Blood Pressure

	Blood pressure (mmHg)	Heart rate (bpm)
Trial #1		
Trial #2		

10/09/2021

Activity Monitor Fitting

☐ Monitor fitted, confirmed to be working
☐ Instructions provided

Maximal Exercise Test (data collection sheet below)
☐ Maximal Exercise Test SOP followed
☐ File labeled:
- Name:

End of Session

☐ Any questions answered
☐ Reminder of date for next session
☐ Instructions for next session provided

10/09/2021

Maximal Exercise Test Data Collection Sheet

Participant Name/Id: Age: Height (cm): Date:

Time (mins)	Speed	Grade	HR	RPE
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				

10/09/2021

A.5. Sessions 2 and 3 Data Collection Sheet

2. **Session 2 or 3: Prolonged or Interrupted Sitting Data Collection Sheet**

Participant No.		**Date**	
Participant Arrival Time		**Session to be Completed Today**	Sitting Interrupted Sitting
Participant Has Arrived Fasted:	YES NO	**Participant Has No New Health Conditions Since Completing the Eligibility Questionnaire:**	YES NO

☐ Participant followed all other instructions
☐ Participant reminded right to withdraw

Resting Measures

Measure	**Data**	**Notes**
Heart Rate		
Blood Pressure		

Saliva Samples
☐ Saliva SOP followed
☐ Eppendorf tubes:
- Number obtained:
- ☐ Labeled

Capillary Samples
☐ Finger prick SOP followed
☐ Eppendorf tubes:
- Number obtained:
- ☐ Labeled

☐ Breakfast provided

Participant Fitted with Strain Gauge:

10/09/2021

Time Chart

Enter the time at which each of the following steps took place:

Event	Time	Notes
Breakfast Provided		
Experiment Began		
Snack Provided		
Experiment Completed		

In-session Measures

Measurement	Measure	Data
1	Heart Rate Blood Pressure	
2	Heart Rate Blood Pressure	
3	Heart Rate Blood Pressure	
4	Heart Rate Blood Pressure	

Post-Session Measures

Measure	Data	Notes
Heart Rate		
Blood Pressure		

Saliva Samples
☐ Saliva SOP followed
☐ Eppendorf tubes:
- Number obtained:
- ☐ Labeled

Capillary Samples
☐ Finger prick SOP followed

10/09/2021

☐ Eppendorf tubes:
- Number obtained:
- ☐ Labeled

Strain Gauge Removed:

Date and time of next session confirmed: YES

10/09/2021

A.6. Saliva Samples Standard Operating Procedures

SALIVA SWAB SAMPLE COLLECTION AND HANDLING

Purpose and Background

Collection and handling of saliva swab samples used for analysis of inflammatory cytokines.

Equipment and Materials

- Oral Swab
- Swab Storage Tube
- Medical Gloves
- Centrifuge
- Freezer
- Cryogenic Gloves
- Biohazard Bag
- Lab Coat
- Paper Towel
- Disinfecting wipes
- 70% alcohol spray

Sample Collection Instructions:

1. If sample is being collected in ACE, cordon off area using long tables in the transition area outside the small chamber, and place signage in the area to indicate biohazardous materials are present.
2. Provide participant with water to rinse their mouth and wait 10 minutes before sample collection.
3. Researcher puts on lab coat, medical gloves, and mask.
4. Researcher removes Oral Swab from packaging.
5. Researcher labels sample.
6. Instruct participant to look at picture and place the swab in their cheek.
7. Instruct participant to keep the swab in place for 5 minutes and to avoid moving the swab around.
8. Instruct participant to remove cap of swab storage tube and place swab inside.
9. Instruct participant to recap swab storage tube.
10. Centrifuge sample (balanced with 2 samples, for 5 minutes, at 1200 rpm).
11. Pipette sample into Eppendorf tubes.
12. Place sample in freezer (-80°C).
13. At the end of each day, wipe down sink with disinfecting wipes.

Participant Instruction

Participants will be instructed on how to perform the saliva collection using this graphic/researcher demonstration.

***Storage**:* Samples will be collected in either UAB 346 or the ACE, and stored in a -80°C freezer in UAB 346. Samples will be transported from the basement of the Science Building to the 4th floor of the Science Building in room UA 4480. Prior to transportation, all samples will be securely sealed and double bagged with the Biohazard Symbol on the outside of the second bag. When samples are collected at the ACE, they will be double bagged and placed in a bucket of ice until the time of transportation; a Biohazard symbol will be placed on the outside of the container used to transport the samples.

***Analysis**:* All samples will be handled in a level 2 (L2) Biosafety Cabinet in UA 4480. All individuals involved with analysis have completed L2 Biosafety Training, and will be added to the appropriate biosafety permits.

***Sample Disposal**:* Saliva samples will be disposed using the UOIT pre-approved protocol as outlined in the Biosafety Manual. All collection tubes, assay plates, plastic pipettes, and gloves will be disposed of in a biohazardous waste bag before autoclaving for at least 30 minutes at 121^{0} C. This bag will be placed into a regular garbage bag for disposal after autoclaving.

***Personal Protective Equipment**:* Gloves will be worn while collecting the sample. New gloves will be worn when analyzing the sample. Hands will be washed each time after gloves are removed. Cryogenic gloves will be used to place samples in the -80°C freezer. The researcher will wear a lab coat and gloves when analyzing samples.

Emergency Response Plan

In the event of a biological spill, section 10.0 of the UOIT biosafety manual will be followed. A hard copy of this manual is available in the laboratory and will be taken to the ACE.

The protocol outlined in section 10.2 in the UOIT biosafety manual will be followed in the event of a small spill of saliva. Relevant procedures are outlined below:

- Immediately notify other individuals in the area that there has been a biohazard spill.
- If necessary, block access to the area.
- Individuals involved in the spill should check for possible contamination of clothing, footwear and skin. Any potentially contaminated clothing should be left in the area until cleaned.
- Before beginning cleanup of the spill put on appropriate protective clothing (gloves, lab coats, mask, etc.)
- Identify the area requiring clean up and decontamination.
- Set up a disposal bag to allow easy discarding of contaminated cleanup materials.
- Use absorbent materials (paper or cloth towels) to dry the area where the saliva has spilled. Wipe the area with disinfecting wipes, and then spray with alcohol spray, and wipe again.
- Place the absorbent material into the disposal bag and repeat the decontamination procedure.
- Remove gloves and place them in a clearly marked biohazard disposal bag with the other contaminated materials. Seal the bag and place it inside another marked biohazard bag for disposal.
- Hand hygiene must be performed at the end of a biohazard spill clean up.

It should be noted that in the event of a medical emergency, the researcher has a hard copy of an emergency action plan posted in the laboratory; this will also be taken to the ACE. There is also a phone in the laboratory and the ACE, so that security can be contacted to initiate emergency procedures.
Of note, a trained researcher will always be present for data collection and analysis.

Incident and Emergency Response

All individuals working with biohazardous materials will report all security incidents to the Certificate holder, Biosafety Officer and Campus Security as soon as possible. Security incidents include, but are not limited to, breach of containment, unauthorized removal of pathogens, and unauthorized personnel in restricted areas. Please refer to the UOIT Biosafety Manual for information on biohazard spill response procedures and other emergency procedures (e.g., earthquake, fire).

Emergency Medical Procedure Response

Emergency medical procedures apply when any person is exposed to body fluids, infections or communicable disease. Exposure may be via a cut or puncture wound, mucous membrane contact or non-intact skin contact.

- Wash the exposed site immediately.
- If mucous membrane contact (eyes, nose, mouth), or non-intact skin contact (cuts, rash or dermatitis), flush with water and the nearest faucet or eye wash station (available in the lab).

- Immediately inform the laboratory supervisor.
- Seek prompt medical attention, giving the medical provider details on the agent.
- As soon as possible, the PI **MUST** complete a UOIT Accident/Injury report. This is to be done electronically. This form can be found at http://www.uoit.ca/forms/accidentinjury/

Biosafety Manual

A hardcopy of this SOP and the UOIT Biosafety Manual is available in the laboratory at all times and will be taken to the ACE when data collection is occurring there.

A.7. Study Protocols Overview