Diagnostic and Treatment Approaches to Fibromyalgia Management: Quantification of Peripheral Blood Mononuclear Cell Function and Evaluation of Radial Shockwave Therapy

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Abstract

Fibromyalgia (FM) is a chronic pain disorder that affects many individuals worldwide. The lack of a definitive diagnostic method and highly successful treatment approaches make it an interesting area of research. In the present study, the effectiveness of quantifying cytokine concentrations from stimulated peripheral blood mononuclear cells (PBMCs) in pre-intervention FM patients as a new diagnostic approach for FM, and the effectiveness of radial shockwave therapy (RSWT) as a FM treatment option to improve the function of PBMC cytokine release from pre- to post-interventions were investigated. Evaluating cytokine concentrations released from stimulated PBMCs allows the capacity of PBMCs to release cytokines and the function of the immune system to be assessed. This was done by isolating PBMCs from blood samples of healthy controls (HCs) and FM participants taken before and after receiving the treatment or placebo intervention, dividing the amount of cells in media from each sample into wells of a cell culture plate, and stimulating half of the cells with the mitogen phytohaemagglutinin protein (PHA-P) to release cytokines. The concentrations of interleukins 6 and 10 (IL-6 and IL-10, respectively) released from stimulated and unstimulated PBMCs were determined from the cell culture supernatants in pre-intervention FM participants and HCs. IL-6 and IL-10 concentrations released from stimulated and unstimulated PBMCs were also determined from the cell culture supernatants before and after FM participants received the treatment or placebo intervention. The effective change ratios for both IL-6 and IL-10 cytokines were also calculated by dividing the cytokine concentration released from stimulated PBMCs by the cytokine concentration released from unstimulated PBMCs for each FM participant. The effective change ratios from pre-intervention FM participants were compared to the effective change ratios from HCs. The effective change ratios from FM participants were also compared pre- and post-intervention for the treatment and placebo groups, and to effective change ratios from HCs. The correlation of IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention was also determined for each FM participant. The results indicated there were no significant differences between HCs and FM participants for the concentrations of IL-6 and IL-10 cytokines released from stimulated and unstimulated PBMCs. There were also no significant differences between HCs and FM participants for both the IL-6 and IL-10 cytokine effective change ratios. There were no significant differences between FM participants pre- and post-intervention for the treatment and placebo groups for the concentrations of IL-6 and IL-10.
cytokines released from stimulated and unstimulated PBMCs. There were also no significant differences observed between FM participants pre- and post-intervention for the treatment and placebo groups for both the IL-6 and IL-10 cytokine effective change ratios. A significant positive correlation was observed between IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention for each FM participant. Additionally, the data did not indicate that RSWT is effective at improving PBMC function for individuals with FM to improve PBMC function as there were no significant differences in IL-6 and IL-10 concentrations released from stimulated PBMCs, or in IL-6 and IL-10 cytokine effective change ratios from pre- to post-intervention for treatment or placebo groups that could potentially indicate an improvement in PBMC function. While previous research has demonstrated the effectiveness of quantifying IL-6 and IL-10 cytokine concentrations released from stimulated PBMCs as a FM diagnostic method, the data do not support these conclusions as no significant differences between the IL-6 and IL-10 concentrations released from stimulated PBMCs, or between the IL-6 and IL-10 effective change ratios were seen between FM participants and HCs. Differences between the previous research and present results could possibly be explained by the screening and inclusion criteria used to enroll FM participants into the studies, or the methods used to quantify cytokine concentrations. An unexpected finding was the lower IL-6 concentration released from stimulated PBMCs in HC females compared to males. Lower IL-10 concentrations released from stimulated and unstimulated PBMCs in HC females compared to males were also observed. The IL-6 effective change ratios were significantly higher in HC females compared to males, but there were no significant differences in the IL-10 effective change ratios between males and females. Future research should focus on using strict and consistent FM screening and inclusion criteria to limit variation in the FM population enrolled into studies, determining which FM subgroup responds the best to RSWT treatment to improve PBMC function, utilizing validated cytokine quantification methods, and studying a larger sample of male and female HCs to determine if the findings of sex differences for cytokines released from stimulated PBMCs are supported.
Lay Summary

Fibromyalgia (FM) is a type of condition that causes individuals to experience chronic pain throughout their body and other mental problems. This condition is very diverse and symptoms affect individuals differently. Many individuals do not know they have this condition as there is no proper way to diagnose it. Individuals who are diagnosed with FM often do not have relief from their symptoms as there are no treatments that are known to successfully work. It is thought that the immune system is involved in the cause of this condition, so the present study used cells of the immune system purified from blood samples of individuals with FM for analyses. Concentrations of interleukin-6 (IL-6) and interleukin-10 (IL-10), which are molecules released from immune cells, were analyzed to determine if these concentrations were different between FM participants and healthy individuals. If there were differences between FM and healthy individuals, this could suggest immune cells in individuals with FM were not functioning properly, and this difference could potentially be used to develop a FM diagnostic method. No significant differences in IL-6 or IL-10 concentrations were observed, however, between FM participants and HCs. Radial shockwave therapy (RSWT) was also assessed as a potential treatment option for individuals with FM. FM participants were assigned to either the group receiving the RSWT treatment, or a group that received the deactivated RSWT treatment, but they did not know their group assignment. IL-6 and IL-10 concentrations were measured before and after FM participants completed treatments to determine if changes in IL-6 or IL-10 concentrations were affected by the RSWT treatment. There were no significant differences in IL-6 or IL-10 concentrations after treatment, which suggests that RSWT is not effective at improving PBMC function for individuals with FM. The heterogeneity of FM suggests that RSWT treatment could potentially benefit a certain group of individuals with FM with specific symptoms. Lower IL-6 and IL-10 concentrations in HC females compared to males were also observed, which could indicate sex differences associated with these molecules.
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Chapter 1. Introduction

1.1 Statement of Problem

The use of novel methods for the diagnosis and treatment of chronic pain disorders has become prominent in current research. Fibromyalgia (FM) is a chronic pain disorder that causes a widespread heightened sensitivity to pain in an individual, along with cognitive symptoms including mood and sleep disruptions, fatigue, depression, memory problems, and lack of focus.\(^1\) An individual is usually diagnosed with FM after other conditions with similar symptoms have been eliminated as FM has no definitive diagnosis or treatment methods available.\(^1\) Currently, the most widely used method to identify FM is the American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for FM screening tool that can be used to diagnose FM by evaluating a patient’s symptoms and pain areas.\(^2\) Refer to the “2010 ACR Preliminary Diagnostic Criteria for FM” section in the Appendix for this screening protocol. Many recent FM research studies focus on developing diagnostic and treatment methods to aid in understanding this complex and misunderstood disorder.\(^3,4,5\)

The identification of cytokines as biomarkers for FM is a diagnostic method that has been previously explored.\(^4,6\) Cytokines are important in the immune system response, and changes in their release could indicate impaired immune cell function.\(^7\) In contrast to inconclusive results from studies investigating basal circulating cytokine levels in the blood, analyzing altered concentrations of cytokines released from stimulated peripheral blood mononuclear cells (PBMCs) is a current detection method that is a promising area in FM diagnostic research.\(^3,4,6\) Evaluating cytokine release from stimulated PBMCs can potentially assess the immune system function of FM patients in reaction to a mitogen stimulus.\(^4\) Interleukins 6 and 10 (IL-6 and IL-10, respectively) from stimulated PBMCs are cytokines of interest as their mitogen-stimulated release have been shown in previous studies by Wallace et al. 2015 and Behm et al. 2012 to be impaired in FM patients compared to healthy controls (HCs).\(^3,4,6\) The variations in released IL-6 pro-inflammatory and IL-10 anti-inflammatory cytokine concentrations could contribute to the pain and symptoms experienced by FM patients.\(^4,7\) Moreover, the altered release of cytokines from PBMCs could indicate that PBMC function in FM patients is affected.\(^4,6\)

In addition to determining FM diagnostic methods, effective treatments for FM have also
been elusive. A multimodal treatment approach has been shown to be most effective at reducing FM associated pain and symptoms; however, this can be costly and time consuming as it is tailored to each person. Radial shockwave therapy (RSWT) is currently being studied as a novel FM treatment method due to its previous success in treating musculoskeletal disorders by improving muscle pain. The RSWT device uses biphasic acoustic energy waves to generate low, medium, or high energy to stimulate tissue inflammation, and it is thought to promote healing by causing cells to undergo a regenerative process. The energy generated is transferred from the device applicator to the treatment area on the skin of FM patients. RSWT is regarded as safe and has no serious side effects other than the potential for short term redness, soreness, and tingling in the treated area. The effectiveness of RSWT in other musculoskeletal disorders make it a high-interest area of research for treating FM.

1.2 Significance of Study

There have been research studies analyzing differences in cytokine concentrations from stimulated PBMCs in FM patients compared to HCs. Studies by Wallace et al. 2015 and Behm et al. 2012 identified aberrant mitogen-stimulated released cytokine concentrations from PBMCs in FM patients, including IL-6 and IL-10 cytokines, and Behm et al. 2012 reported their potential as biomarkers for a FM diagnostic method. The use of the widely accepted 2010 ACR Preliminary Diagnostic Criteria for FM in previous studies by Wallace et al. 2015 and Behm et al. 2012 is significant as these criteria were used in the current study to classify FM in participants and verify the measurement of aberrant IL-6 and IL-10 cytokine concentrations released from stimulated FM PBMCs as these previous studies used this criteria and observed lower released cytokine concentrations in FM. The use of enzyme-linked immunosorbent assays (ELISAs) is also significant as its improved ability to accurately detect IL-6 and IL-10 cytokine concentrations released from stimulated PBMCs can be evaluated in contrast to previous research by Behm et al. 2012 and Wallace et al. 2015 who used multiplex immunoassays, which have been criticized regarding their ability to accurately quantify cytokine concentrations.

There is little known on the ability of RSWT to treat FM. RSWT is thought to promote localized repair and healing by stimulating tissue inflammation that causes cells to undergo a regenerative process, which may regulate immune cell function due to an increase in an
individual’s overall well-being from reduced pain.\textsuperscript{5} Measuring deviations in stimulated PBMC cytokine release before and after a FM patient receives RSWT treatments can indicate if RSWT is effective at improving PBMC function and its capacity as a FM treatment option.

1.3 General Research Question

Is RSWT effective at improving PBMC function in individuals with FM?

1.4 Specific Aims

Specific Aim 1. Determine the effectiveness of quantifying cytokine concentrations from stimulated PBMCs in pre-intervention FM participants as a new diagnostic approach for FM.

\textbf{Rationale:}

Cytokine release is part of the body’s immune system response to promote healing, tissue repair, and inflammation.\textsuperscript{7} Certain aberrant cytokine concentrations released from stimulated PBMCs may be associated with chronic pain, and FM patients have been shown in some studies to have altered concentrations when compared to HCs, as shown in Table 1 in Section 2.4.1 of Chapter 2.\textsuperscript{3,4,6,7,9} This difference may indicate that FM patients have an impaired immune function that affects the release of cytokines from PBMCs, which could contribute to incomplete tissue repair and lead to chronic pain.\textsuperscript{4,7} Lower pro-inflammatory and anti-inflammatory aberrant cytokine concentrations in FM patients such as IL-6 and IL-10 from stimulated PBMCs supports the thought of an altered ability for PBMCs to release cytokines.\textsuperscript{4,7} The identification of these altered cytokines as biomarkers for FM could be a potential diagnostic method and indicator of FM treatment success at the molecular level.\textsuperscript{4,6}

\textbf{Hypotheses:}

\textbf{H}_{1a}: IL-6 and IL-10 cytokine concentrations from stimulated PBMCs will be lower in FM participants compared to HCs.
H1b: IL-6 and IL-10 cytokine effective change ratios, which are determined by dividing the cytokine concentration released from stimulated PBMCs by the cytokine concentration released from unstimulated PBMCs for each FM participant, will be lower in FM participants compared to HCs.

Specific Aim 2. Determine the effectiveness of RSWT at improving the function of FM PBMC cytokine release from pre- to post-interventions.

**Rationale:**

RSWT has been used to successfully treat musculoskeletal disorders by stimulating tissue inflammation, which is thought to cause cells to undergo a regenerative process and promote healing. The known effectiveness of RSWT indicates its potential to reduce chronic pain associated with FM by promoting proper and complete localized tissue repair. This action may in turn regulate systemic immune cell function, including the altered release of cytokines from stimulated PBMCs in FM patients, due to an increase in an individual’s overall well-being from reduced pain. It has been shown in studies by Lin et al. 2018 and Cyranowski et al. 2007 investigating depression and cytokine concentrations, that an altered release of cytokines from stimulated PBMCs may be associated with depression and accompanying symptoms. Another study by Weizman et al. 1994 examining a clomipramine drug treatment to increase suppressed cytokine production from stimulated PBMCs in majorly depressed patients showed an improvement in depression symptoms and an increase in suppressed cytokines after treatment. An improvement in depression symptoms could increase an individual’s overall well-being, which may in turn regulate cytokine production. Since depression can occur with FM, it can be suggested that treatments improving FM associated pain may increase an individual’s overall well-being, which may subsequently regulate PBMC function and cytokine release. Tests and questionnaires were administered to FM participants pre- and post-intervention to assess their pain levels including a visual analog scale to assess pain levels, the Baseline Pressure Tolerance Meter, the Quantitative Sensory Testing for hyperalgesia and allodynia, the Beighton Scoring Screen for hypermobility, and the Pain Catastrophizing Scale. These measurements were analyzed in another arm of the study. RSWT can be evaluated as a FM treatment option by measuring deviations in cytokine concentrations released from PBMCs
before and after a FM patient completes all the treatments. Circulating cytokines differ from cytokine released from stimulated and unstimulated PBMCs as cytokines released from PBMCs can be used to assess the immune system function of an individual with FM. Reduced IL-6 and IL-10 released from stimulated PBMCs in individuals with FM compared to HCs could indicate an altered FM PBMC function. Differences in cytokine release can also indicate the effectiveness of RSWT at promoting tissue repair and improving PBMC function. An improvement in FM associated pain could potentially regulate PBMC function and cytokine release due to an increase in an individual’s overall well-being.

**Hypothesis:**

H2: FM participants in the RSWT treatment group will have increased post-intervention IL-6 and IL-10 cytokines released from stimulated PBMCs compared to those in the placebo group.
Chapter 2: Literature Review

2.1 Characterization of Fibromyalgia (FM)

FM is widely accepted as a non-inflammatory chronic pain disorder; however, there is some evidence to suggest it could be an inflammatory disorder.\(^1\)\(^,\)\(^6\)\(^,\)\(^8\) FM causes an individual to experience a widespread heightened sensitivity to pain, along with cognitive symptoms including mood and sleep disruptions, fatigue, depression, memory problems, and lack of focus.\(^1\) While there are many symptoms associated with FM, almost all individuals with FM experience fatigue, pain, and sleep disturbances.\(^13\) It has also been shown that individuals with FM have increased blunt pressure and heat stimuli sensitivities, which could indicate aberrant central pain processing.\(^14\) The intensity and location of FM associated pain can frequently change in an individual’s body depending on various factors or for unknown reasons.\(^15\) FM affects approximately 2-5% of the general adult population worldwide, and more commonly occurs in women compared to men with a prevalence of 3.4% compared to 0.5%, respectively.\(^16\)\(^,\)\(^15\) An increased sensitivity to pain in women causing a greater response to painful stimuli could explain the higher occurrence of FM in women compared to men.\(^16\) Although individuals with FM can range from teenagers to older adults, FM most commonly occurs in middle-aged individuals.\(^15\)

2.1.1 Classification and Identification

Currently, FM has no clear diagnostic methods. Individuals with FM are usually diagnosed after eliminating other conditions with similar symptoms due to a lack of definitive diagnosis and treatment methods available for FM.\(^1\) Diagnosing an individual with FM may involve the coordination of various specialists, with a definitive diagnosis often being made by a rheumatologist.\(^15\) The uncertain diagnostic criteria for FM makes an accurate diagnosis challenging and also contributes to increased costs for health care as tests to rule out other conditions must be performed, and various treatment methods must be attempted.\(^17\) Only criteria based on the analysis of symptoms and comorbid disorders in an individual are available to identify FM, which is not highly reliable.\(^16\)

The most widely used method to classify FM is the American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for FM.\(^2\) This is a screening tool that evaluates a patient’s
symptoms and pain areas to classify but not definitively diagnose FM, which decreases its reliability.\textsuperscript{2} The original 1990 ACR criteria for classifying FM differs from the revised 2010 ACR criteria as they focus on identifying a minimum number of tender points and widespread pain without addressing cognitive symptoms associated with FM.\textsuperscript{18} The 2010 criteria focus on a patient’s symptoms and pain locations instead of identifying tender points since the use of tender points is not a highly reproducible or reliable method to classify FM.\textsuperscript{18} Both criteria are still widely used in the literature to identify individuals with FM for FM related studies. The ACR Preliminary Diagnostic Criteria for FM is currently the most accepted method to identify FM, but there are some disadvantages associated with it. In addition, this screening tool can also only identify symptoms that occur more severely in an individual, which may misclassify some individuals with different FM subtypes or who only have mild symptoms.\textsuperscript{18} An advantage of this screening tool is that it can provide a method to investigate potential FM comorbidities and their relationship to FM.\textsuperscript{18}

The lack of definitive diagnostic criteria for FM is concerning due to the relatively high reported prevalence of individuals with FM in society. Research investigating the spectrum of FM could lead to the development of more effective diagnostic criteria that could potentially identify FM subgroups. With an increased knowledge of FM diagnosis through research and education, individuals with FM can be diagnosed more effectively.\textsuperscript{19}

\textbf{2.1.2 Hypotheses for Etiology and Pathogenesis}

The pathogenesis of FM has been a high interest area of research, but the etiology of this disorder is still not clearly known.\textsuperscript{16,15} Previous research suggested many hypotheses which could contribute to the creation and development of FM, including the role of infectious agents such as hepatitis C and hepatitis B, and gene analysis studies evaluating an individual’s potential predisposition for FM.\textsuperscript{15} In addition to researching different agents that could lead to FM, identifying potential deregulations of certain systems in the body and their interaction with other systems has also been studied. One thought is that FM could partly be due to altered pain processing in the central nervous system (CNS), which causes an increased sensitivity to pain perception known as hyperalgesia and allodynia.\textsuperscript{16} The CNS in individuals with FM could also be hyperactive from an irregular perception and processing of painful stimuli in neurotransmitter systems.\textsuperscript{15} Research has shown that FM is partly related to stress, and that chronic stress could
cause alterations in neurotransmitter systems in individuals with FM.\textsuperscript{20} It is also thought that central sensitization may be involved in FM, where the nervous system overreacts from receiving various pain stimuli and causes chronic pain to develop in an individual.\textsuperscript{21,22} As a result of central sensitization, pain signal transmission and neuron stimulation in the CNS are increased.\textsuperscript{23}

Although there are many hypotheses for the etiology and pathogenesis of FM, the connection between the nervous system and immune system of individuals with FM has been examined. A study by Staud 2015 investigated if dysfunctional interactions are present in the nervous and immune systems of individuals with FM that could potentially lead to FM.\textsuperscript{24} The importance of the immune system in the pathogenesis of FM has been recognized due to the altered levels of immune mediators discovered from previous studies, including one from Behm et al. 2012.\textsuperscript{4} It is also known that both the immune and nervous systems use cytokine signaling molecules, which allows the systems to communicate with each other directly.\textsuperscript{24} Since these systems are associated with analgesia and pain symptoms in an individual, their interactions with each other are of interest as these symptoms are also associated with FM.\textsuperscript{24} While dysfunctions in the CNS and immune system are thought to be the main contributors to FM associated pain and symptoms, the involvement of potential dysfunctions from the autonomic nervous and endocrine systems have also been studied in individuals with FM.\textsuperscript{25} By understanding the mechanisms of defective central pain processing in the nervous system for FM, the etiology and pathogenesis of this disorder can be further developed and studied.\textsuperscript{23}

\subsection*{2.1.3 Subgroups}

Although FM is recognized as a chronic pain disorder that causes a widespread heightened sensitivity to pain along with cognitive symptoms, it is not a single homogeneous condition.\textsuperscript{1,26,27} There is evidence of FM subgroups where individuals have different sensitivities to certain pain stimuli and various degrees of other cognitive symptoms.\textsuperscript{26} The differences in FM associated symptoms between individuals also indicates that some subgroups will respond better to certain treatments compared to other subgroups. The concept of FM subgroups is supported by the knowledge that pain, symptoms, and comorbid conditions associated with FM are highly variable between individuals.\textsuperscript{26} Specific pain and symptom characteristics can be identified for each FM subgroup, which could be used to determine the best combination of treatment methods to use, as various FM subgroups respond better to different treatments.\textsuperscript{26} Giesecke et al. 2003
analyzed mood, cognition, and hyperalgesia of individuals with FM and classified individuals into three FM subgroups. This study found individuals in the first FM subgroup had an extreme sensitivity to pain without any cognitive or mood symptoms, the second subgroup had a moderate sensitivity to pain without any abnormal mood symptoms, and the third subgroup had the greatest sensitivity to pain with extreme cognitive and mood symptoms. It has also been acknowledged that some FM subgroups can be identified based on the presence or absence of depression as this comorbidity can greatly affect the treatment method of an individual with FM.

The ACR Preliminary Diagnostic Criteria for FM screening tool is currently the most widely used method to diagnose FM; however, it is unable to solely identify different FM subgroups. Analyzing FM associated symptoms using a variety of methods could also create different classification criteria for FM subgroups, which could make diagnosing and treating FM more difficult. The knowledge that FM can be further classified into subgroups based on differences in pain severity, cognitive symptoms, and comorbid inflammatory and non-inflammatory conditions is beneficial as it can allow a unique combination of treatment methods for individuals to be created. By tailoring treatments to an individual’s specific somatic and psychological symptoms, there is a greater chance for treatment effectiveness and success for improving FM associated symptoms. A customized approach for treating individuals with FM could be effective at reducing specific FM symptoms associated with each subgroup, and to identify potential inflammatory FM subgroups.

2.2 Comorbid Conditions with FM

Pain and cognitive dysfunctions, including sleep disturbances and fatigue, are the most common FM associated somatic and cognitive symptoms. FM is known to be comorbid with certain other conditions that may also have common FM associated symptoms, especially mood and anxiety disorders. A comorbidity indicates that one or several other conditions may be present in an individual along with FM. Since these conditions may share some of the same symptoms as FM, this could make identifying each one challenging. A number of conditions that are comorbid with FM may also be classified as functional somatic syndromes, which are syndromes that are related and identified based on an individual’s symptoms instead of by functional variations. Specifically, conditions that may occur along with FM have been referred
to as central sensitivity syndromes due to their association with chronic pain and the central nervous system.\textsuperscript{1,21,22}

2.2.1 Physical Pain Conditions

While there are numerous conditions that are comorbid with FM, chronic fatigue syndrome (CFS) is one condition that has been shown to be highly comorbid with FM.\textsuperscript{22,17,13} Both FM and CFS have common symptoms such as fatigue, widespread pain, sleep disturbances, and cognitive dysfunctions that makes distinguishing between the two conditions challenging.\textsuperscript{13} To further associate CFS and FM, a considerable number of individuals with FM have been shown to meet the CFS criteria, and some individuals with CFS have also met the FM criteria.\textsuperscript{18} The association of an increased sensitivity to a stimulus for several shared symptoms between individuals with FM and CFS may provide a potential common mechanism for these two conditions.\textsuperscript{31} In addition to CFS, chronic widespread pain (CWP) is considered to be a main condition that is also comorbid with FM.\textsuperscript{18,32}

Individuals with FM experience a heightened sensitivity to pain throughout their entire body, particularly observed more in the arms, shoulders, and lower back, which coincides with CWP symptoms.\textsuperscript{18,32} The cause of CWP is unclear; however, it is suggested that its mechanism may be similar to that of FM.\textsuperscript{32,18} Rheumatoid arthritis (RA) is another condition that has also been shown to be comorbid with FM.\textsuperscript{33,25} Unlike FM, RA causes chronic pain and swelling in the joints.\textsuperscript{33} Despite having different inflammatory reactions in the body, both conditions share the commonality of causing pain to an individual.\textsuperscript{33} Irritable bowel syndrome (IBS) is another well-known comorbidity of FM, and it causes abdominal pains and discomfort.\textsuperscript{34} Individuals with IBS have an increased gut hypersensitivity comparable to the hyperalgesia sensations that are characteristic of FM.\textsuperscript{22} Individuals with IBS experience somatic hypoalgesia, however, which is opposite to individuals with FM who experience heightened somatic pain.\textsuperscript{35} An individual with FM and an IBS comorbidity may have CWP that includes the abdomen area.\textsuperscript{35}

2.2.2 Cognitive Dysfunction Conditions

In addition to comorbid conditions of FM that cause physical pain, there are also comorbid conditions that cause cognitive dysfunctions. Depression is a cognitive disorder that is well-known to be associated with chronic pain conditions and has a high comorbidity with
It has been stated in the literature that individuals with FM are found to have depression more often than any other FM comorbidity. The reason for the high prevalence of depression as a FM comorbidity has been hypothesized, but no clear link has been established. One hypothesis is that an individual with FM could develop depression due to living with chronic pain and having a decreased overall well-being. Another hypothesis is that the etiology of both FM and depression share a common mechanism that causes both conditions to develop in an individual with FM. Similar to hyperalgesia sensations associated with FM, individuals with symptoms of depression may also experience a greater perception to pain; however, there is no definitive link between individuals with depression and altered pain processing as this could be affected by various aspects. FM individuals experiencing depression are also likely to experience anxiety conditions, such as post traumatic stress disorder (PTSD). Although the link between FM and anxiety conditions is not fully established, the association of experiencing traumatic events with the development of physical pain has been observed.

Diagnostic tests to distinguish FM from other potentially comorbid conditions can be useful to investigate connections between these conditions. This could aid in developing FM treatment methods that address multiple symptomatic and cognitive aspects of FM, and allows the potential to treat FM and its comorbid conditions present in an individual using various intervention methods. Overall, the recognition of certain comorbid conditions with FM could contribute to an increase and improvement in FM diagnosis and treatment, respectively.

### 2.2.3 Mast Cell Activation Syndrome (MCAS)

Mast cells (MCs) are a type of granulated cell found in connective tissue that are important in the immune system response, and they release substances including histamine, heparin, and inflammatory mediators. Certain MC dysfunctions can cause MC disorders, including MCAS and mastocytosis. MCAS is the improper activation of MCs from immunologic and non-immunologic methods, which can result in aberrant amounts of cellular mediators being released from MCs such as inflammatory cytokines. MCAS does not involve the over-proliferation of MCs in certain tissues, which is seen in mastocytosis. MC dysfunctions are also thought to be the underlying cause of many conditions such as obesity, asthma, CFS, IBS, and FM. MCs have previously been thought to be involved with FM partly due to their association with pain perception in individuals. Studies by Afrin 2016, Blanco et
al. 2010, and Enestrbm et al. 1997 found an increased amount of activated MCs located in the uppermost dermis layer of skin biopsies from FM individuals that could be linked to this condition’s etiology and pathogenesis.\textsuperscript{41,43,44} Since MCs are close to neurons in many areas of the body, it was hypothesized that increased amounts of corticotrophin-releasing hormone (CRH) and substance P (SP) released from neurons cause the activation of localized MCs and release of neurosensitizing and pro-inflammatory mediators.\textsuperscript{40,43} This action could affect the peripheral and central nervous system of some FM individuals, which could influence FM associated pain and symptoms.\textsuperscript{45}

It has been suggested in the literature that MCAS may be comorbid with FM.\textsuperscript{39,46} Many symptoms for MCAS overlap with symptoms for FM including fatigue, depression, memory problems, mood disturbances, and cognitive dysfunctions.\textsuperscript{38,39} A major symptom seen in MCAS is migratory pain areas, which is similar to the type of pain experienced by individuals with FM.\textsuperscript{39,45} Like FM, MCAS has no definitive diagnostic criteria, which makes it difficult to identify in individuals.\textsuperscript{47} Although MCAS could be comorbid with FM, the causes of both disorders are not clearly understood.\textsuperscript{47} MCAS has also been suggested to be comorbid with other conditions including hypertension, asthma, and postural orthostatic tachycardia syndrome (POTS), which indicates the complexity of this syndrome.\textsuperscript{39} Overall, current research demonstrates there could be a link between MCAS and FM; however, more research is needed to fully develop a clear connection between MC dysfunction, MCAS, and FM.

2.3 Peripheral Blood Mononuclear Cell (PBMC) Function in FM

2.3.1 Phytohaemagglutinin protein (PHA-P) Stimulation and PBMC Activation

Different mitogens can be used to stimulate various cell types, such as lipopolysaccharide (LPS) to stimulate B-cell proliferation.\textsuperscript{48} PHA-P is a type of mitogen lectin that is used to stimulate PBMCs to release cytokines.\textsuperscript{4} It has been used in previous FM research by Behm et al. 2012 and Wallace et al. 2015 to stimulated isolated PBMCs, and it is used in the present study to verify the results obtained from the previously mentioned FM research.\textsuperscript{3,4} It is the protein form of phytohaemagglutinin (PHa) that is extracted from the red kidney bean \textit{Phaseolus vulgaris}.\textsuperscript{49} PHA-P is composed of the two isolectin subunits phytohaemagglutinin erythrocyte (PHA-E) and phytohaemagglutinin leukocyte (PHA-L) that interact with erythrocytes and leukocytes,
respectively. PHA-E allows erythrocytes to agglutinate, and PHA-L agglutinates and acts as a mitogen for lymphocytes. Since PBMCs isolated from peripheral blood contain no erythrocytes, PHA-P can be used as the PHA-E subunit will not affect the mitogenic properties of the PHA-L subunit to stimulate PBMCs. If red blood cell contamination is present in the isolated PBMCs, PHA-E will agglutinate the erythrocytes and it will not affect the mitogenic properties of PHA-L.

PHA-P is a polyclonal activator protein that stimulates and activates naïve T-cells that have clonally distributed surface antigen receptors known as T-cell receptors (TCRs). TCRs contain α and β heterodimers that bind the antigen of interest, and the CD3 complex consists of four invariant signaling chains that are specific for the different T-cell antigen binding receptors, as shown in Figure 1. PHA-P activates T-cells by attaching to glycoproteins, as well as TCRs and CD3 complexes specific to certain antigens that are both located on the T-cell surface plasma membrane. The glycoproteins and CD3 complex are cross-linked by the binding of PHA-P to the T-cell surface, causing T-cell activation, proliferation, and cytokine release. PHA-P attaches to T-cell surface receptors instead of receptors on other lymphocytes as TCRs contain sugar moieties that are recognized by the mitogen. PHA-P mimics an immune response in vitro by acting like a general antigen that activates T-cells in a PBMC extraction. This action can evaluate PBMC immune function by determining cytokine secretion profile patterns of T-cells following stimulation.
TCRs contain α and β heterodimers that bind the antigen of interest. The CD3 complex consisting of four invariant signaling chains that are specific for clonally distributed TCRs. The polyclonal mitogen activator phytohaemagglutinin protein (PHA-P) binds to the TCR and cross-links glycoproteins and the CD3 complex on the T-cell surface to activate the T-cell. Adapted from Franco et al. 2016.

Although PHA-P can only stimulate T-cells, all PBMCs are incubated with PHA-P as it has been shown that monocytes need to be present as accessory cells for PHA-P to stimulate T-cells since isolated T-cells do not proliferate only in the presence of PHA-P. This is due to the monocyte-derived helper activity where factors in monocyte culture supernatant such as interleukin-6 (IL-6), aid in T-cell stimulation and proliferation by PHA-P, and factors from stimulated T-cells aid in monocyte stimulation. Since PHA-P can only stimulate T-cells, its use is limited if all PBMCs need to be stimulated including monocytes, B-cells, and natural killer (NK) cells. The mitosis of lymphocytes other than T-cells does not occur using PHA-P as this mitogen is either inactive or inhibits the process for these cells. It is known that PBMCs consist of around 10-20% monocytes and 70-90% lymphocytes. The lymphocyte population is made up of 5-20% NK cells, 5-10% B-cells, and 70-85% CD3+ T-cells. Within the T-cell population exists a 2:1 ratio of CD4+ helper T-cells and CD8+ cytotoxic T-cells. Since some PBMC T-cell subpopulations can be determined based on their cytokine secretion patterns, it may be
possible to identify certain T-cell subpopulation dysfunctions by analyzing cytokine concentrations in response to stimulation. The ability of PHA-P to successfully bind to T-cell glycoproteins, TCRs, and CD3 complexes and activate T-cells can influence cytokine concentrations as proper binding is needed in part for activation. If these domains are altered, this could affect T-cell activation and cytokine release. Despite other cell types being present in the PBMC extraction along with T-cells, the exclusive stimulation of T-cells by PHA-P was chosen to expand on previous research by Behm et al. 2012 and Wallace et al. 2015. This method of stimulation was used in the previous research to analyze PBMC function and evaluate the immune response of individuals with FM. Although T-cells are the most predominant cell type in the PBMC extraction, other PBMC types in individuals with FM may experience altered functions that might not be detected using this method. Evaluating the adaptive immune response is important for determining a diagnostic method for FM as it could indicate potential PBMC dysfunctions and altered cytokine release patterns unique to FM individuals.

2.3.2 PBMC Dysfunction in Other Conditions

In addition to research studying potential altered PBMC function by quantifying released cytokine concentrations in FM patients, there have also been studies done on other conditions that exhibit an aberrant cytokine release response from PBMC stimulation, which could indicate PBMC dysfunction. Wallace et al. 2015 compared IL-6, interleukin-8 (IL-8), macrophage inflammatory protein-1 alpha (MIP-1α), and macrophage inflammatory protein-1 beta (MIP-1β) cytokine concentrations from stimulated PBMCs in healthy controls (HCs) to FM, RA, and systemic lupus erythematosus (SLE) patients and found a unique cytokine profile for FM. All four cytokine concentrations were lower in FM individuals compared to HC, RA and SLE patients except for IL-8 which had a similar concentration to RA. Mean cytokine/chemokine composite test scores calculated from combined cytokine concentrations for each FM, RA, SLE, and HC group determined that 93% of FM individuals had a positive test score for identifying FM, compared to 11% for HCs, 31% for RA, and 29% for SLE. These findings indicated this test score method for determining a cytokine profile had a 93% sensitivity and 89.4% specificity for a FM diagnosis compared to HCs. These findings also suggest that PBMC function may be altered in FM patients as well as other systemic inflammatory autoimmune processes, and that cytokine expression differences could potentially be used as a differential diagnosis method.
Some conditions that exhibit an aberrant cytokine response from PBMC stimulation and potential PBMC dysfunction are also seen in individuals with FM, including depression, chronic pain, and CFS. It is known that improperly functioning cytokines are involved in the pathogenesis of depression. Lin et al. 2018 analyzed PBMC function in patients with major depressive disorder (MDD) and reported lower interleukin-2 (IL-2) and interleukin-10 (IL-10) cytokine release from stimulated PBMCs in these individuals compared to HCs. Weizman et al. 1994 analyzed the production of interleukin-1 beta (IL-1β) and interleukin-3-like activity (IL-3 LA) cytokines from PBMCs in major depressed patients before and after treatment with clomipramine. This study found that initially suppressed cytokine levels in major depressed patients compared to HCs increased after treatment, along with an improvement in depression in these individuals. Gür et al. 2002 examined the concentrations of serum cytokines interleukin-1 (IL-1), IL-2 receptor (IL-2r), IL-6, and IL-8 in individuals with FM and their relationship to depression using the Hamilton Depression Rating Scale (HDRS) and observed higher serum IL-8 and IL-2r, and HDRS scores in individuals with FM compared to controls. Another study by Zou et al. 2018 investigating cytokine levels from the blood serum of patients with MDD also found abnormal cytokine levels compared to HCs, which could be associated with potential PBMC dysfunction in MDD patients.

Individuals with chronic pain have also been shown to have increased IL-1β expression from stimulated PBMCs, which could indicate an altered ability for PBMCs to release cytokines. The transforming growth factor beta 1 (TGF-β1) cytokine is important for inflammatory characteristics of CFS, and deregulations in its release from stimulated PBMCs was also observed in CFS individuals. Since the potential relationship between PBMC dysfunction and aberrant cytokine responses from PBMC stimulation has been studied and recognized in conditions that can also occur with FM, it can be suggested that PBMC dysfunction may be a commonality underlying FM and associated conditions that could be linked to the pathogenesis of FM.

2.3.3 Sex and Age Influences

There are differences reported in the literature between concentrations of cytokines released from stimulated PBMCs in HCs and individuals with FM, but there is minimal research analyzing sex and age differences of cytokine concentrations in HCs.
concentration differences from stimulated PBMCs between HC males and females over a range of ages is important as variations could provide more insight into the pathogenesis and potential PBMC dysfunction of FM. There have been studies analyzing the role of cytokines and pain perception in individuals which have not investigated potential cytokine concentration differences between sexes and only used entire male or female populations to eliminate possible variations that could arise. Euteneuer et al. 2011 analyzed the serum concentrations of tumor necrosis factor alpha (TNF-α) in patients with major depression and observed higher concentrations in patients compared to HCs, but there were no significant differences between sexes in either of the patient or HC groups. In depressed patients, however, there was a correlation between a decrease in pressure pain threshold and increase in TNF-α concentration in women but not in men. The findings from this study could support previous research identifying variations in chronic pain perception between sexes based on sex differences in the immune system instead of the nervous system. This study could also aid in the development of diagnostic and treatment methods for FM due to the comorbid association of depression with FM.

In addition to a lack of research analyzing sex differences for cytokine concentrations and pain perception in individuals with depression, there is also minimal research for the influence of age and sex on cytokine concentrations released from stimulated PBMCs in HCs. There have been some studies using basal cytokine concentrations to analyze the relationship of cytokines to age and/or sex; however, studies using cytokines released from stimulated PBMCs are scarce. Verthelyi et al. 2000 analyzed the correlation between various sex hormone levels and the number of PBMCs secreting IL-2, interleukin-4 (IL-4), IL-6, IL-10, TNF-α, and interferon gamma (IFN-γ) in vivo. It was found that secreted IL-4 correlated with oestrogen hormone levels during the menstrual cycle, and secreted IFN-γ correlated with dehydroepiandrosterone sulfate (DHEA-S) hormone levels in males. These results suggest that cytokine production by PBMCs may be influenced by sex hormones, which could contribute to differences in immune responses based on sex. It also provides support for the potential influence of sex hormones on immune function due to certain factors, including women having a higher susceptibility to autoimmune diseases. There is also evidence that the adaptive immune response is affected by age, as observed by the reduction of T-cell numbers and increase in basal cytokine concentrations in older individuals; however, this does not provide a direct assessment of
immune system function. Evaluating the ability of immune cells, such as T-cells, to be stimulated and release cytokines can assess immune system function by determining if these cells are releasing cytokines properly, and if the released cytokine levels are altered.

Sturgeon et al. 2014 investigated basal cytokine concentrations in serum samples from pre- and post-menopausal women with FM to determine if hormone differences due to age influenced cytokine production in females. It was observed that the DHEA-S hormone of interest did not correlate with cytokine concentrations in pre- or post-menopausal women, which could be due to basal cytokine levels being measured instead of levels from stimulated PBMCs. This study, however, did not compare cytokine differences with age in males or in healthy pre- and post-menopausal women to determine if similar or variant cytokine patterns could be seen. With little research on cytokine differences over various ages and between sexes, this study suggested that hormonal differences between males and females could partially influence cytokine concentrations. This study also acknowledged that research is lacking regarding cytokine concentration changes with age in individuals with FM.

There is also evidence that age and sex could affect cytokine production from monocytes and T-cells. Pietschmann et al. 2003 evaluated cytokine release from stimulated PBMCs in young and elderly males and females, and it was observed that age influenced T-cell cytokine production; elderly women had increased levels of IL-4 and IFN-γ from stimulated CD8+ T-cells compared to young women. It was also found that elderly men had increased levels of IL-2, IL-4, and interleukin-13 (IL-13) released from T-cells compared to young men. Between sexes, certain cytokines released from stimulated T-cells, such as IFN-γ and IL-10, had changes specific for each sex. Overall, more research looking at differences in other cytokines released from stimulated PBMCs between sexes is needed to find potential causes for male and female cytokine concentration differences.

Without knowing the cause of potential variations in cytokine release from stimulated PBMCs for each sex across different ages, research evaluating differences in immune response could be confounded. Future research analyzing cytokine differences in males and females at various ages would be beneficial for many research disciplines attempting to identify and use cytokines as potential biomarkers for disease diagnosis, including FM diagnosis.
2.4 Diagnostic Methods for FM

2.4.1 FM/a® Test

A functional biomarker challenge test (FBCT) can detect regulatory differences of a certain biomarker of interest, and it can be used to evaluate the function of the immune system. The FM/a® test is a blood test that is reported to objectively diagnose FM, and it is marketed by the company EpicGenetics. This test uses a multi-biomarker approach to detect altered cytokine concentrations released from stimulated PBMCs, and irregular cytokine patterns compared to controls can identify individuals with FM. An individual is diagnosed with FM based on a scoring system for the cytokines of interest, where a score of 50 or higher on a 1-100 scale indicates a FM diagnosis.

The method for the FM/a® test to identify altered cytokine concentration patterns from stimulated PBMCs in individuals with FM and distinguish them from HCs was studied and developed by Behm et al. 2012 and Wallace et al. 2015. Behm et al. 2012 observed eight cytokine concentrations from stimulated PBMCs that were altered in individuals with FM compared to controls, and a follow up study by Wallace et al. 2015 observed that four of the eight altered cytokines could identify individuals with FM from individuals with RA and SLE. The study by Behm et al. 2012 observed seven of the eight cytokines analyzed had significant differences in released cytokine concentrations from stimulated PBMCs in individuals with FM compared to HCs, with the exception of the interleukin-5 (IL-5) cytokine which did not have a significant difference. The study by Wallace et al. 2015 observed three of the four released cytokine concentrations from stimulated PBMCs analyzed were significantly different in individuals with FM compared to HCs and individuals with autoimmune disorders, with the exception of the IL-8 cytokine which was not significantly different. The mean range of select altered cytokine concentrations released from stimulated PBMCs highlighted by Behm et al. 2012 and Wallace et al. 2015; and the range of minimum to maximum values of released cytokine concentrations, and cytokine median detection ranges from Behm et al. 2012 for individuals with FM and HCs are shown in Table 1. The IL-6 and IL-10 cytokines exhibited the greatest difference between HCs and individuals with FM in the Behm et al. 2012 study, where individuals with FM had a blunted response to cytokine release.
Table 1. Mean cytokine concentrations released from stimulated PBMCs in individuals with FM and HCs.

The released cytokine concentration ranges from minimum to maximum values are shown for individuals with FM and HCs from Behm et al. 2012. Mean concentration values are taken from papers by Behm et al. 2012 and Wallace et al. 2015. Median detection ranges in pg/mL for each cytokine are taken from Behm et al. 2012. Concentrations are in pg/mL. Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Median Detection Range</th>
<th>Individuals with FM</th>
<th>HCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Range</td>
<td>Concentration</td>
</tr>
<tr>
<td>IL-6</td>
<td>1 to 33000</td>
<td>276 ± 437 to 2667 ± 1149</td>
<td>2799 ± 4182 to 5365 ± 1901</td>
</tr>
<tr>
<td>IL-8</td>
<td>2 to 60000</td>
<td>5751 ± 6123 to 17298 ± 7094</td>
<td>17456 ± 24246 to 10433</td>
</tr>
<tr>
<td>IL-10</td>
<td>2 to 63000</td>
<td>12 ± 15 &lt;OOR to 84</td>
<td>80 ± 94 &lt;OOR to 352</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>1 to 7000</td>
<td>204 ± 272 to 1104 ± 465</td>
<td>1084 ± 1773 to 1876 ± 777</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>5 to 14000</td>
<td>2900 ± 2417 to 10756 ± 4350</td>
<td>4413 ± 5228 to &gt;OOR</td>
</tr>
</tbody>
</table>

Abbreviations: FM – Fibromyalgia, HCs – Healthy Controls, IL – Interleukin, MIP-1α – Macrophage Inflammatory Protein-1 Alpha, MIP-1β – Macrophage Inflammatory Protein-1 Beta, OOR – Out of Range, PBMCs – Peripheral Blood Mononuclear Cells, SD – Standard Deviation

In Table 1, the range of minimum to maximum values of released cytokine concentrations from Behm et al. 2012 were considerably large, but significance was still obtained for all cytokines except IL-5, which had a p value of 0.136 (significant p value range for other cytokines was from <0.001 to 0.016). In addition, the results from Behm et al. 2012 and the Wallace et al. 2015 strongly suggest detecting altered cytokine responses from stimulated PBMCs could be used as a FM diagnostic method; however, research produced from these papers may be questioned. The principal investigator on both papers is the owner of
EpicGenetics, and other authors are employees for the company. This relationship can potentially question the validity of the FM/a® diagnostic method for FM.

The FM/a® test is also compliant with the United States Food and Drug Administration (FDA) under the Code of Federal Regulations (CFR) Title 21, Part §866, Subpart F – Immunological Test Systems 5700 – Whole human plasma or serum immunological test system. Although the FM/a® test is compliant with the FDA and regulations of the CFR, this does not imply that it is also FDA approved or regulated. An FDA regulated product indicates that a product approval, processing, and use in the commercial market is overseen by the FDA. By recognizing the relationship between EpicGenetics company employees and their published papers that use methods from the FM/a® test, and the knowledge of the FDA compliant instead of FDA regulated status of the FM/a® test, the importance to independently confirm the findings of using altered cytokine concentrations released from stimulated PBMCs as a diagnostic method for FM is established.

2.4.2 Cytokines as FM Biomarkers

Cytokines have been studied as potential biomarkers for diagnosing FM. Cytokines are proteins released from cells and identified as either anti-inflammatory or pro-inflammatory as they respectively cause a decrease or increase in inflammation throughout the body from a response to a stimulus. They are responsible for cell communication, including cells in the immune and nervous systems. Changes in cytokine release could indicate impaired immune cell function, which could affect the immune system response and contribute to the pathogenesis of chronic pain in FM patients. PBMCs are cells of the immune system that release cytokines upon activation in vivo with cytokines from an immune response, or upon stimulation in vitro with a mitogen stimulus. Stimulating PBMCs and analyzing the released cytokine concentrations can be used to assess immune system function in individuals with FM. Studies by Wallace et al. 2015 and Behm et al. 2012 analyzing released cytokine concentration differences from stimulated PBMCs in FM patients compared to HCs found FM patients had aberrant cytokine concentrations. Although altered cytokine concentrations are observed in FM patients, there is a lack of consensus regarding their increased or decreased regulation compared to HCs. It is presumed, however, that pro- and anti-inflammatory cytokine imbalances are associated with pain initiation and maintenance.
There have also been studies investigating serum or plasma basal circulating cytokine levels in the blood of FM patients, but the results have been inconclusive and do not directly assess immune system function.\textsuperscript{3,4,6} These varying results could partially be due to diurnal effects of cytokine production as it has been observed that specific cytokines are produced in particular amounts at certain times during the day within the body.\textsuperscript{71} It has also been observed that individuals with FM have cytokine changes throughout the day in plasma, which could affect results obtained from various studies depending on when blood samples are collected from participants.\textsuperscript{72} A summary of various studies analyzing cytokine concentrations from stimulated or unstimulated immune cells in blood samples for individuals with FM is shown in Table 2. A variety of techniques were used to process the blood samples and different methods were used to quantify cytokine concentrations, which makes direct comparisons between these studies not possible.
Table 2. Cytokine concentrations from stimulated or unstimulated immune cells in blood samples for individuals with FM.

For each study, the FM diagnostic criteria used to include FM participants, types of immune cells analyzed, types of stimulation, method used to quantify cytokine concentrations, cytokines investigated, and results obtained from comparing cytokine levels in FM participants to HCs were examined.

<table>
<thead>
<tr>
<th>Diagnostic Criteria</th>
<th>a) Immune Cells</th>
<th>Cytokines</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM diagnosis &gt;1 year, ACR 1990 and 2010</td>
<td>a) PBMCs</td>
<td>IL-5, IL-6, IL-8, IFN-γ, IL-10, MIP-1α, MIP-1β, MCP-1</td>
<td>More cytokines released from stimulated PBMCs vs. unstimulated; less cytokines released from stimulated PBMCs of FM patients vs. HCs</td>
<td>Behm et al. 2012.4</td>
</tr>
<tr>
<td>FM diagnosis &gt;1 year, ACR 1990 and 2010</td>
<td>a) PBMCs</td>
<td>IL-6, IL-8, MIP-1α, MIP-1β</td>
<td>Lower cytokine concentrations released from stimulated PBMCs of FM patients vs. HCs</td>
<td>Wallace et al. 2015.3</td>
</tr>
<tr>
<td>Rheumatologist FM diagnosis, ACR 1990</td>
<td>a) Monocytes</td>
<td>Eotaxin, MDC, GRO-α</td>
<td>More eotaxin, MDC, and GRO-α released from stimulated and unstimulated monocytes of FM patients vs. HCs</td>
<td>García et al. 2016.73</td>
</tr>
<tr>
<td>ACR 1990</td>
<td>a) PBMCs</td>
<td>IL-1β, IL-2, IL-6, IL-8, IL-10</td>
<td>Increased IL-1RA and IL-6 cytokine concentrations from stimulated and unstimulated</td>
<td>Wallace et al. 2001.74</td>
</tr>
<tr>
<td>Criteria of general physical pain symptoms outlined by Smythe, 1979</td>
<td>a) CD4+ T-lymphocytes, T-lymphocytes</td>
<td>IL-2</td>
<td>More mitogen needed for T-lymphocytes from FM patients to secrete comparable amount of IL-2 from HCs</td>
<td>Hader et al. 1991.75</td>
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</tr>
<tr>
<td>Rheumatologist FM diagnosis, ACR 1990</td>
<td>a) PBMCs</td>
<td>IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α</td>
<td>Higher IL-2 concentration released from stimulated PBMCs in FM patients vs. HCs pre-DEX; greater reduction in cytokine concentrations in FM patients vs. HCs after DEX treatment</td>
<td>Macedo et al. 2007.76</td>
</tr>
<tr>
<td>Rheumatologist FM diagnosis, ACR 1990</td>
<td>a) Monocytes</td>
<td>IL-1β, TNF-α, IL-6, IL-10</td>
<td>Higher cytokine concentrations released from stimulated and unstimulated monocytes in FM patients vs. HCs pre-aquatic exercise; comparable cytokine concentrations in FM patients and HCs post-aquatic exercise</td>
<td>Ortega et al. 2012.77</td>
</tr>
</tbody>
</table>

**Abbreviations:** ACR – American College of Rheumatology Preliminary Diagnostic Criteria for FM, Con A – Concanavalin A, DEX – Dexamethasone, ELISA – Enzyme Linked Immunosorbent Assay, FM – Fibromyalgia, GRO-α – Growth Regulated Oncogene, HCs –...
Healthy Controls, IFN-γ – Interferon Gamma, IL – Interleukin, IL-1RA – Interleukin 1 Receptor Antagonist, LPS – Lipopolysaccharide, MCP – Monocyte Chemoattractant Protein, MDC – Macrophage Derived Chemokine, MIP-1α – Macrophage Inflammatory Protein-1 Alpha, MIP-1β – Macrophage Inflammatory Protein-1 Beta, PBMCs – Peripheral Blood Mononuclear Cells, PHA – Phytohaemagglutinin, PMA – Phorbol Myristate Acetate, sIL-2R – Serum IL-2 Receptor, TNF-α – Tumor Necrosis Factor Alpha

Overall, there was no general consensus regarding an increase or decrease in pro-inflammatory or anti-inflammatory cytokine concentrations in individuals with FM compared to HCs after immune cells were stimulated with a mitogen or if they were unstimulated. However, there was a trend for studies by Behm et al. 2012, Wallace et al. 2015, and Hader et al. 1991, analyzing cytokines released from stimulated T-cells to have lower released cytokine concentrations in individuals with FM compared to HCs.3,4,75 There was also a trend for studies by García et al. 2016, Wallace et al. 2001, and Ortega et al. 2012, analyzing cytokines released from stimulated monocytes to have higher released cytokine concentrations in individuals with FM compared to HCs.73,74,77 These trends could be due to the different types of stimulation methods and cell types used for each study. Despite different quantification methods used to detect various released cytokine concentrations from stimulated immune cells for studies in Table 2, Harder et al. 1991 observed lower released IL-2 concentrations in individuals with FM compared to HCs.75 This agrees with results from Behm et al. 2012 and Wallace et al. 2015 identifying lower released cytokine concentrations from stimulated PBMCs in individuals with FM compared to HCs.3,4,75 The four studies in Table 2 by García et al. 2016, Wallace et al. 2001, Macedo et al. 2007, and Ortega et al. 2012 observed higher released cytokine concentrations from stimulated immune cells in individuals with FM compared to HCs, which were opposite to the results from the Wallace et al. 2015 and Behm et al. 2012 studies.3,4,73,74,76,77 The results from all studies in Table 2 indicated there was evidence of a potentially altered immune cell function in individuals with FM compared to HCs.

In addition to the previously observed trends for lower released cytokine concentrations from stimulated T-cells, and higher released cytokine concentrations from stimulated monocytes in individuals with FM compared to HCs, the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 released from stimulated PBMCs are of particular interest as their
concentrations were observed by Behm et al. 2012 and Wallace et al. 2015 to be lower from stimulated T-cells in FM patients compared to HCs, and they were observed by Wallace et al. 2001 and Ortega et al. 2012 to be higher from stimulated monocytes in FM patients compared to HCs.\(^3\)\(^,\)\(^4\)\(^,\)\(^74\)\(^,\)\(^77\) These irregular cytokine concentrations could contribute to FM associated chronic pain as it has been shown that certain cytokines are associated with pathological pain.\(^7\) IL-6 is associated with hyperalgesia, depression, and fatigue, which are some symptoms of FM, while IL-10 is correlated with blocking pain signals in the body.\(^74\) Identifying cytokine deregulations could potentially be used as a biomarker for FM, which could aid in the development of a FM diagnostic method.

### 2.4.3 Multiplex and Enzyme Linked Immunosorbent Assay (ELISA) Cytokine Detection Methods

There are different methods to detect cytokine concentrations from serum or plasma samples, including ELISAs or multiplex assays. Both the ELISA and multiplex methods have been shown to have exceptional specificity and sensitivity for effectively detecting a variety of proteins, including cytokines.\(^78\) These methods are widely used and accepted in the literature; however, there are some advantages and disadvantages associated with each. The ability of a multiplex assay to detect numerous cytokines from a single sample has greatly improved experimental processing time, and helped researchers gather the most information from experiments. The multiplex assay is more cost effective compared to an ELISA if multiple cytokines need to be quantified for each sample as less reagents and laboratory supplies are consumed.\(^78\) The assay also requires less sample volume, which is advantageous for research studies with limited sample quantities. Despite the many advantages of multiplex assays, their use in clinical settings is not widely accepted as only a small number of assays have been approved for use.\(^79\) Compared to ELISAs, multiplex assays require stricter quality control criteria to ensure the simultaneous measurements of numerous cytokines are accurate.\(^79\) There is also a concern for cross-reactivity between target cytokines and capture and detection antibodies, which can affect proper multiplex assay functioning and potentially cause results to be misrepresented.\(^79\) Antibodies used in ELISAs may also not work if combined in a multiplex assay due to the potential for cross-reactivity’s to occur.\(^79\) By analyzing capture antibodies and
their interactions with specific cytokine targets and other antibodies, improvements can be made to obtain accurate results from multiplex assays.\textsuperscript{79}

Although multiplex assays have many advantages, there are also some advantages for ELISAs. The ability of ELISAs to quantify a single cytokine of interest in a sample can be useful for diagnosing certain diseases that are confirmed by the detection of a single cytokine.\textsuperscript{79} ELISAs have also been recognized as the top validated method for cytokine detection.\textsuperscript{78} There is also only a single antibody being used to detect a certain cytokine, so there is minimal potential for cross-reactivity to occur and the cytokine measurement obtained is more accurate. Despite the many advantages of ELISAs, they are unable to diagnose more complex diseases requiring the detection of numerous cytokines for an accurate diagnosis.\textsuperscript{79} ELISAs are also not as cost-effective or efficient as multiplex assays.\textsuperscript{78} With the technology for multiplex assays being improved for use in clinical settings, ELISAs are still considered the gold standard for accurate cytokine detection and measurement.\textsuperscript{78}

2.5 Treatment Methods for FM

FM has been widely classified as a chronic pain disorder with cognitive function impairments as well as hypersensitivity in an individual due to altered pain perception and response from certain stimuli.\textsuperscript{15} Unlike acute pain, most medication and other non-pharmacologic treatment methods are ineffective at fully alleviating chronic pain and associated symptoms on their own.\textsuperscript{15} The heterogeneity and complexity of FM makes finding effective treatments difficult as there is no single treatment method that works for all individuals with FM.\textsuperscript{1,16} It has been observed that a combination of treatment methods provides the best outcome for alleviating pain and symptoms associated with FM.\textsuperscript{1} These treatment options can be tailored to each individual and range from using a pharmacological and symptom-based treatment approach, to a non-pharmacological and holistic approach.\textsuperscript{21,19} Due to the many factors that contribute to the cause of pain, a variety of medications with different mechanisms of action in the body may have to be used for successful pain management.\textsuperscript{23}

2.5.1 Pharmacological and Non-Pharmacological Approaches

The study by Bonaccorso et al. 1998 observed the use of selective serotonin reuptake inhibitor (SSRI) and tricyclic antidepressant medications to improve FM associated somatic and
cognitive symptoms in some individuals, including pain and hyperalgesia responses, and depression and sleep disturbances, respectively.\textsuperscript{36} The percentage of individuals who experienced a significant improvement in FM associated pain and symptoms from taking antidepressants, however, is only around 10 to 25\%.\textsuperscript{80} The use of antidepressants may not be effective to treat all individuals as FM is a heterogeneous spectrum disorder with different subtypes, which require individualized treatments. In addition to pharmacological treatments, non-pharmacological methods are also recommended to treat FM including low intensity aerobic exercise programs, cognitive behavioural therapy, healthy lifestyle choices, and FM associated symptom coping strategies.\textsuperscript{15,19,21} A drawback of cognitive behavioural therapy is that it may only improve the cognitive functioning of an individual and not directly decrease physical pain.\textsuperscript{15}

\subsection*{2.5.2 Anti-inflammatory and Immunosuppressant Medications}

Non-steroidal anti-inflammatory drugs (NSAIDs) are a type of medication used to treat individuals with inflammation and pain. They work by inhibiting the cyclooxygenase-1 and 2 (COX-1 and COX-2) enzymes that are mostly present in the endoplasmic reticulum of cells that produce prostanoid to reduce their production of prostaglandin, which subsequently reduces pain, inflammation and fever in specific injured tissue areas throughout the body.\textsuperscript{81,82} The effectiveness of NSAIDs and other natural anti-inflammatory compounds have been studied by Lu et al. 2018 and Tanaka et al. 1998 with \textit{in vitro} models of inflammation using PBMCs stimulated with a mitogen.\textsuperscript{83,84} In these studies, cytokine concentrations were measured before and after the addition of an NSAID to stimulated PBMCs to determine if the drug had an effect at reducing pro-inflammatory cytokine concentrations.\textsuperscript{83,84} These studies demonstrated the anti-inflammatory abilities of NSAIDs and their effects on the immune response as seen by a reduction of pro-inflammatory cytokine levels.\textsuperscript{83,84} Ceuppens et al. 1986 also observed that the NSAID naproxen taken orally for seven days improved lymphocyte proliferation following stimulation with PHA in RA patients due to an immunomodulatory effect of the medication.\textsuperscript{85} There are, however, no known studies analyzing the effectiveness of NSAIDs in FM, or analyzing the \textit{in vivo} effects of NSAIDs on PBMCs after being isolated from blood.

In addition to NSAIDs, opioids are a stronger type of medication also used to manage pain. They work by interacting with opioid receptors in the central nervous system to block painful stimuli and produce analgesia.\textsuperscript{82} Opioid analgesic treatment has previously been used to
help control FM pain levels, although its long term use is now discouraged by many medical guidelines due to its lack of effectiveness when compared to other treatment options such as various nonpharmacologic therapies and FDA approved pharmacotherapies. A pilot study by Parkitny et al. 2017 using the low dose naltrexone opioid receptor antagonist in individuals with FM observed improvements in FM associated pain and symptoms and reductions in pro-inflammatory cytokine plasma concentrations. However, there has been no clinical trial research studies that have proven the effectiveness of opioids to treat FM. Overall, since FM is widely accepted as a non-inflammatory chronic pain disorder, NSAIDs and opioids are not recommend to treat FM associated pain. They can successfully treat most peripheral pain complaints, but are not effective for treating central pain disorders such as FM.

In addition to the ineffective use of NSAIDs and opioids to treat FM, there is also evidence that immunosuppressant medications are not beneficial as a treatment method for FM. Different immunosuppressants, such as glucocorticoids, work by interacting with specific biological targets in the body to cause immune function changes in an individual. These medications have anti-inflammatory and immunosuppressive properties that inhibit inflammatory and immune responses in the body. Although immunosuppressants can reduce inflammation and aid in managing rheumatic conditions, their ability to weaken the immune system and cause infection are side effects that make them undesirable for treating FM. Overall, immunosuppressant therapy for individuals with FM is not effective as FM is widely accepted as a non-inflammatory chronic pain disorder.

2.5.3 Radial Shockwave Therapy (RSWT)

RSWT is a potentially novel FM treatment method that has been successful in treating musculoskeletal disorders such as chronic tendinopathies, but there is little research on its ability to treat FM. Some musculoskeletal disorders reported in which shockwave therapy has successfully treated are shown in Table 3.
Table 3. Examples of musculoskeletal disorders shockwave therapy has successfully treated.

<table>
<thead>
<tr>
<th>Musculoskeletal Disorder</th>
<th>Type of Shockwave Treatment Used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofascial Pain Syndrome</td>
<td>Focused Low-Energy ESWT</td>
<td>5</td>
</tr>
<tr>
<td>Proximal Plantar Fasciitis</td>
<td>ESWT</td>
<td>92</td>
</tr>
<tr>
<td>Lateral Epicondylitis of the Elbow</td>
<td>ESWT</td>
<td>92</td>
</tr>
<tr>
<td>Calcific Tendinitis of the Shoulder</td>
<td>ESWT and RSWT</td>
<td>92,93</td>
</tr>
<tr>
<td>Achilles Tendinopathy</td>
<td>ESWT</td>
<td>92</td>
</tr>
<tr>
<td>Patellar Tendinopathy</td>
<td>ESWT</td>
<td>92</td>
</tr>
<tr>
<td>Delayed and Non-Union of Long Bone Fractures</td>
<td>ESWT</td>
<td>92</td>
</tr>
</tbody>
</table>

Abbreviations: ESWT – Extracorporeal Shockwave Therapy, RSWT – Radial Shockwave Therapy

RSWT is also known as radial pressure waves as the treatment shockwave intensity is very low and a true shockwave it not generated.\textsuperscript{5,93} RSWT and extracorporeal shockwave therapy (ESWT) function by generating low to medium, or high biphasic acoustic energy waves from the device, respectively.\textsuperscript{5} This energy is transferred from the applicator to the treatment area on the skin of FM patients.\textsuperscript{5} RSWT and ESWT differ from each other due to the physical characteristics of pressure and energy densities of their waves over time.\textsuperscript{93} RSWT has a longer rise time and lower peak pressure compared to ESWT.\textsuperscript{93} The radial shockwaves used in RSWT are generated by a projectile accelerating in the device, which are then transferred radially from the device applicator to the treatment area.\textsuperscript{93} The focused shockwaves used in ESWT are generated in the device applicator using an electrohydraulic technique, or externally to the treatment area using piezoelectric or electromagnetic techniques, which are then transferred in a focused manner to the specific treatment area.\textsuperscript{5,93} RSWT has an unfocused application, where ESWT has a focused application with respect to the treatment area on the skin of FM patients.\textsuperscript{93} There are no safety concerns with the RSWT device and it only has potential mild side effects including short term redness, soreness, and tingling in the treated area.\textsuperscript{5} A benefit of RSWT is it can be administered
to an individual without anesthesia as it uses lower energy shockwaves and an unfocused application, which make it is less painful compared to ESWT.\textsuperscript{93}

RSWT is thought to cause cells to undergo a regenerative process and promote healing through localized tissue repair by stimulating tissue inflammation using the mechanical effects of the RSWT device to cause disruptions in the tissue.\textsuperscript{5} Tissue repair is achieved due to primary and secondary effects from shockwaves being administered to a treatment area.\textsuperscript{92} The primary effect of shockwaves generated from the device is the production of direct mechanical forces acting on the tissue in the treatment area, and the secondary effect of shockwaves is cavitation from indirect mechanical forces.\textsuperscript{92} The bursting of cavitation bubbles creates a force that can stimulate cells to undergo tissue healing. This physical process causing tissue inflammation in FM patients may potentially reduce FM associated chronic pain, which could regulate immune cell function from an increase in an individual’s overall well-being.\textsuperscript{5}

Overall, the use of various treatments to create unique multimodal approaches for FM management has currently been shown to have the greatest effect at improving an individual’s FM associated pain and symptoms.\textsuperscript{21,19} The inclusion of RSWT as part of an FM treatment program can act as a pain modulator in addition to using other FM treatment methods and components. The effectiveness of RSWT at improving FM associated pain and symptoms can continue to be studied to determine if it can be a potential FM treatment option.
Chapter 3: Methodology and Experimental Design

3.1 Research Ethics Board (REB) Study Approval

The present study has been reviewed and approved by the Thunder Bay Regional Health Sciences Centre (TBRHSC), St. Joseph’s Care Group (SJCG), and Lakehead University (LU) REBs. Approvals have also been obtained from the REBs for changes made to the original protocol.

3.2 Experimental Design

3.2.1 Participant Recruitment

Potential participants were recruited using flyers advertising the study. These flyers were posted at local clinics and organizations that have a high population of individuals with fibromyalgia (FM). A study advertisement was placed in the local paper to reach more individuals. Electronic advertising was achieved using social media and online clinic newsletters. Interested potential participants contacted the research team using the information on the recruitment flyer or advertisement to arrange an informed consent and screening meeting.

3.2.2 Participant Informed Consent and Screening

The informed consent and screening meeting determined if potential FM and healthy control (HC) participants were eligible to enroll in the study. Refer to the “Flow of FM Participants through Each Stage of the Present Study” section in the Appendix for the flow chart outlining this process. Potential HC participants were screened and met the inclusion criteria as follows: they must have been between 18-60 years of age, willing to talk with a research team member and provide informed consent, and able to speak and read basic English. They also had to meet the exclusion criteria as follows: they must not have been pregnant, had no current anti-inflammatory use, and not be diagnosed with thrombosis, thrombophlebitis, or coagulation disorders which could cause an increased health risk during blood draws. Smoking, diabetes, or body mass index were not screened for in potential participants, and these factors could alter immune function.
Potential FM participants must have met the inclusion criteria as follows: they must have been between 18-60 years of age, willing to talk with a research team member, able to speak and read basic English, and willing to give the name of their primary healthcare provider/physician. They must have also met the exclusion criteria as follows: they must have had no vascular, neurological or other condition or disorder that could explain their pain, had no implanted metal that was incompatible with shockwave therapy, not be pregnant, and not be diagnosed with thrombosis, thrombophlebitis, or coagulation disorders which could have caused an increased health risk during blood draws. Potential FM participants were screened using the 2010 American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for FM to ensure they were eligible to participate. If a potential FM participant did not meet either the inclusion, exclusion, or 2010 ACR Preliminary Diagnostic Criteria for FM, they were not permitted to partake in the study. Ineligible participants had their informed consent and screening forms marked as a “Screen Fail”, and these were kept separate from forms of approved participants.

Potential FM and HC participants could take time to consider participating in the study, and ask questions before providing informed consent. Participants consented to the study by signing the informed consent form. Participants could withdraw from the study at any time without reason. Participants that were withdrawing could choose to have any or all data collected from them destroyed, with the exception of the screening and informed consent forms which must be kept for a minimum of five years. The duration of participation in the study for FM individuals was between six to seven consecutive weeks.

3.2.3 Participant Enrollment

FM participants who passed the screening criteria and provided informed consent were enrolled into the study. Each FM and HC participant was assigned a unique number code to ensure their identity remained anonymous during data collection and analysis. Baseline measures from the FM participants were taken pre- and post-intervention using a variety of tests and questionnaires to assess pain levels. These tests could be used to determine if possible sub-groups of FM individuals were present, which could influence an individual’s response to the radial shockwave therapy (RSWT) treatment. A visual analog scale allowed FM participants to rate their pain levels from three of the most painful areas in different regions on their body. The Baseline Pressure Tolerance Meter determined the pressure pain threshold in lbs of the three
painful areas by having increasing pressure applied to each area until the FM participant reported pain.\textsuperscript{95} The Quantitative Sensory Testing for hyperalgesia and allodynia quantified the FM participant’s increased sensitivity to pain from the three painful areas using a standardized methodology.\textsuperscript{96} The Beighton Scoring Screen for hypermobility assessed motions done on certain areas of a FM participant’s body to determine if hypermobility was present.\textsuperscript{97} The Pain Catastrophizing Scale evaluated a FM participant’s pain levels and feelings towards their pain.\textsuperscript{98} The sex and age of FM and HC participants were recorded, as well as the mass, height, and current anti-inflammatory medication use for FM participants.

3.2.4 Participant Group Randomization

In this randomized controlled trial study, participants were randomly assigned to either the study treatment or placebo group based on their sex and age. This randomized group assignment process is called minimisation, and it was used to remove bias from group assignment and to ensure equal group sizes were obtained.\textsuperscript{99} The QMinim online program was used to randomize participants as it utilizes this process.\textsuperscript{100} Participants were told their group assignment after they completed the study. Participants who were randomized into the placebo group had the RSWT treatment offered to them for free after the official study and data collection was completed. No data was collected or used from participants during the free RSWT treatments as this data would not be part of the original study design. The individual administering the interventions knew all group assignments to ensure each participant received the appropriate treatment.

3.2.5 Treatment

The type of intervention for each participant was administered based on their group assignment. Participants in the treatment group received the active form of the RSWT treatment, which was administered as 500 shocks (1.5 bar, 15 Hz), followed by 1000 shocks (2 bar, 8 Hz), and then another 500 shocks (1.5 bar, 15 Hz). Participants in the placebo group received a similar treatment, but a different device applicator with a soft rubber cap was used that caused an air gap between the participant’s skin and transmitter. The placebo intervention was administered as 500 shocks (0.01 mJ/mm\textsuperscript{2}, 15 Hz), followed by 1000 shocks (0.01 mJ/mm\textsuperscript{2}, 8 Hz), and then another 500 shocks (0.01 mJ/mm\textsuperscript{2}, 15 Hz). The Storz Duolith® SD1 Ultra Shockwave module
device (Storz Medical AG, Tägerwilen, Switzerland) was used to administer the active or placebo RSWT intervention to each of the three most painful areas attributed to FM in different regions on the participant’s body. The intervention sessions occurred once weekly for five weeks. At the start of each session, changes to the participant’s anti-inflammatory or pain medication use were also noted.

3.2.6 Blood Sample Collections

A blood sample was collected at the LU School of Kinesiology Sanders Building from the participant before beginning and after completing the five weeks of interventions. A blood sample was collected from HC participants only once as they did not participate in the intervention sessions. A phlebotomist drew one vial of blood for each of the two visits using specialized 4 mL draw capacity BD Vacutainer® CPT™ Tubes with Sodium Citrate and a density solution (Becton, Dickson and Company, Franklin Lakes, New Jersey, USA, cat #362760), and other supplies needed for a blood draw. The specialized vacutainers contained an anti-coagulant that did not allow the blood to clot. Following centrifugation, plasma was formed and all the blood components were suspended, including a distinct layer of peripheral blood mononuclear cells (PBMCs). This process allowed PBMCs to be isolated from the participant’s whole blood. Each vial of blood was labeled with the participant’s number code and “A” or “B”, which indicated if the blood was drawn before or after receiving treatments, respectively. The blood samples were transported to the Northern Ontario School of Medicine (NOSM) laboratory for processing and analysis.

3.2.7 Blood Sample Processing and Storage

Once the vacutainer containing the participant’s blood sample was transported to the NOSM laboratory, it sat at room temperature for one to two hours before processing. The vacutainer was then inverted eight to ten times to re-mix the blood sample before being placed into a centrifuge machine with vacutainer adapters and water balance to spin for 25 minutes at 1500 G-force (X g). The centrifuge separates PBMCs from whole blood due to the centripetal force generated that causes higher density blood components to travel to the bottom of the vacutainer quicker than less dense blood components. Following centrifugation, the clear layer of PBMCs located below the plasma layer and above the density layer solutions was removed from
the vacutainer, as indicated in the BD Vacutainer® CPT™ Tube with Sodium Citrate information booklet (Becton, Dickson and Company, Franklin Lakes, New Jersey, USA). The PBMCs were transferred to a 15 mL conical centrifuge tube, and phosphate buffered saline (PBS) modified with calcium and magnesium (GE Healthcare Life Sciences, South Logan, Utah, USA, cat #SH30264.01) was added to a final volume of 15 mL. The tube was inverted five times before being placed into a centrifuge with conical centrifuge tube adapters and water balance for 15 minutes at 300 x g to wash the PBMCs of unwanted blood components. The supernatant was then poured off, the pellet was resuspended by gently tapping the tube, and PBS was added to a final volume of 10 mL for another wash step. The tube was inverted five times before being placed into a centrifuge with conical centrifuge tube adapters and water balance for 10 minutes at 300 x g.

During this time, supplemented Roswell Park Memorial Institute (RPMI) 1640 media (2.05 mM L-Glutamine, 10% fetal clone III (FCIII), 1% penicillin-streptomycin, 1% sodium pyruvate (NaPyr); GE Healthcare Life Sciences, South Logan, Utah, USA, cat #SH3002701, refer to “RPMI Media Preparation” section in the Appendix for the recipe), and the mitogen phytohemagglutinin-P (PHA-P; Sigma-Aldrich, St. Louis, Missouri, USA, cat #L1668) were warmed in the bead heater. Following centrifugation, the supernatant was poured off, and the pellet was resuspended in 1 mL of media. 20 µL of this resuspension was used for a 10-fold dilution with 180 µL of PBS. Ten µL of the 10-fold dilution was transferred to each side of a hemocytometer to count the PBMCs and determine the number of cells per mL in the resuspension. Based on the number of cells in the resuspension, the appropriate amount of media was added to dilute the resuspension and obtain one million cells per mL. No media was added if the resuspension already contained less than one million cells per mL. The final resuspension volume was divided equally into two wells of a cell culture plate labeled “stimulated” and “unstimulated”. PHA-P was diluted to a final concentration of 10 µg/mL and added to the “stimulated” well. PHA-P was used for stimulation as it was previously used in FM studies by Behm et al. 2012 and Wallace et al. 2015 analyzing the release of cytokines from stimulated PBMCs, and the present study is verifying the results obtained from these previous studies.³,⁴ The seeded cells were incubated at 37˚C with 5% carbon dioxide (CO₂) for 18 hours.

After the incubation period, the PBMCs in media were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 10 minutes at 16000 x g at 4˚C. The supernatant from
each sample was pipetted into new 1.5 mL microcentrifuge tubes and inverted to mix. Based on the volume of each sample, 220 µL and 30 µL aliquots were made in 1.5 mL microcentrifuge tubes. These tubes were labeled with the date, participant code and if the sample was taken pre- or post-interventions. They were stored at –80°C. Refer to the “Blood Sample Processing and Storage” section in the Appendix for the full standard operating procedure (SOP).

3.2.8 Blood Sample Preparation for Enzyme-Linked Immunosorbent Assay (ELISA)

The processed blood samples stored at –80°C were prepared before being used in an ELISA analysis. Based on the standard range detection limit of ELISA kits, the samples were diluted to ensure the biomarker of interest was detected. The Human Interleukin-6 (IL-6) ELISA MAX™ Deluxe set (BioLegend, San Diego, California, USA, cat #430504) required samples stimulated with PHA-P to be diluted to allow IL-6 concentrations to fall within the standard range of 7.8-500 pg/mL. After the samples were removed from the -80°C freezer and placed on ice to thaw, serial dilutions were done to allow IL-6 concentrations to fall within the middle of the standard curve for each sample. The dilutions used for data collection varied within the serial dilution range depending on the sample. A 10-fold dilution was initially done, followed by a 5-fold dilution, a 3-fold dilution, and a 2-fold dilution. All dilutions were plated to ensure IL-6 concentrations were detected if there was variability between samples. The Human Interleukin-10 (IL-10) ELISA MAX™ Standard set (BioLegend, San Diego, California, USA, cat #430601) required samples stimulated with PHA-P to be diluted to allow IL-10 concentrations to fall within the standard range of 3.9-250 pg/mL. After the samples were removed from the -80°C freezer and placed on ice to thaw, serial dilutions were done to allow IL-10 concentrations to fall within the middle of the standard curve for each sample. The dilutions used for data collection varied within the serial dilution range depending on the sample. A 10-fold dilution was initially done, followed by two 2-fold dilutions. All dilutions were plated to ensure IL-10 concentrations were detected if there was variability between samples. Unstimulated PBMC supernatant samples did not need to be diluted as the full sample concentration was low enough to potentially be detected within the IL-6 or IL-10 standard range. The samples were diluted using 1X Assay Diluent A from the BioLegend ELISA kits, which was 1X PBS solution with bovine serum (BioLegend, San Diego, California, USA, cat #421203). Aliquoted stimulated and unstimulated PBMC supernatant samples were only thawed and used once to avoid potential effects on
cytokine concentrations caused by repeated freeze and thaw cycles. Refer to the “Blood Sample Preparation for Enzyme-Linked Immunosorbent Assay (ELISA)” section in the Appendix for the full SOP.

3.2.9 Blood Sample Analysis using ELISA

To measure the IL-6 and IL-10 concentrations in the PBMC stimulated and unstimulated supernatant samples, Human IL-6 and IL-10 ELISA MAX™ sets (BioLegend, San Diego, California, USA) were used following the manufacturer’s directions. The standard dilution series, blank and samples were plated in duplicate, and 100 µL of each were loaded into the wells of a Nunc™ MaxiSorp™ flat-bottom 96 well uncoated ELISA plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA, cat #44240421). The plate was read at 450 nm and 570 nm on a BioTek Power Wave XS microplate reader (model #MQX200R) using KC4 analysis software, and the delta optical density (OD) absorbance readings were recorded. Refer to the “Blood Sample Analysis using ELISA” section in the Appendix for the full SOP.

3.3 Limitations, Basic Assumptions, and Delimitations

3.3.1 Limitations and Basic Assumptions

In the present study, there were limitations and assumptions associated with the participation of FM and HC individuals. One limitation was that FM participants might not be an accurate representation of the entire FM population due to the limited location where study treatments could be administered, a limited participant recruitment of 13 female individuals with FM, and multiple-week commitment to the study. It was assumed the ACR Preliminary Diagnostic Criteria for FM was reliable at accurately classifying FM to identify potential study participants. It was also assumed FM participants did not have other underlying health conditions not captured on the screening criteria that made them ineligible to participate or that could interfere with the study results. It was assumed the baseline measure tests and questionnaires completed pre and post intervention for FM participants, including the visual analog scale, the Baseline Pressure Tolerance Meter, the Quantitative Sensory Testing for hyperalgesia and allodynia, the Beighton Scoring Screen for hypermobility, and the Pain Catastrophizing Scale, were reliable and accurate at assessing pain levels. For HC
participants, it was assumed that those who passed the screening criteria were in good health and
did not have underlying health conditions that could affect the study results. Another assumption
was that FM and HC participants answered all screening questions truthfully. A limitation was
that participants might have experienced different stressors in their lives that could have
contributed to variations in blood draw analysis and treatment results. It was assumed the
randomization software used to assign FM participants to the placebo or treatment group was
operating properly. It was also assumed the RSWT device was functioning as expected to deliver
the appropriate treatment to FM participants. It was assumed the blood collection and analysis
materials were not defective and the results obtained from them were accurate.

3.3.2 Delimitations

In the present study, cytokine supernatants of stimulated and unstimulated PBMCs
isolated from human blood samples of FM and HC participants were used for all experiments.
Participants were classified as having FM but did not necessarily have a FM diagnosis from a
rheumatologist. This potentially allowed more individuals to be included in the study as there are
no definitive FM diagnostic methods available. FM and HC participants also passed their
respective study inclusion and exclusion criteria to ensure they were eligible to participate. These
criteria addressed potential safety concerns, as well as factors that could impact study results.
Potential other factors that were not screened for that could impact the study include smoking,
diabetes and an individual’s body mass index as these factors could potentially affect immune
function. Another delimitation was the variation of blood draw and RSWT treatment or placebo
intervention times for each FM participant. This was done to accommodate each participant’s
schedule. All treatments and blood draws were performed in the same conditions and rooms to
minimize variability. Participants were recruited from specific locations around the city,
including local clinics, SJCG Chronic Pain Management and Rheumatic Disease Programs, and
the 55 Plus Centre. These locations were chosen as they had a high population of individuals
with FM.

3.4 Statistical Analysis

To answer the hypotheses from Specific Aim 1, separate one-tailed Student’s t-tests for
IL-6 and IL-10 cytokines were used to compare the effective change in stimulated and
unstimulated cytokine concentrations from pre-intervention FM participants and HCs. Separate one-tailed Student’s t-tests for IL-6 and IL-10 cytokines were also used to compare stimulated and unstimulated cytokine concentrations from pre-intervention FM participants and HCs. Stimulated to unstimulated ratios were determined for each participant before determining the means when comparing the stimulated and unstimulated samples.

To answer the hypothesis from Specific Aim 2, separate two-way repeated measures analysis of variances (ANOVAs) for IL-6 and IL-10 cytokines were used to compare the effective change in stimulated and unstimulated cytokine concentrations from FM participants pre- and post-treatment or placebo interventions. The Fisher’s least significant difference (LSD) post-hoc test was used as two separate group means can be directly compared to each other. Separate two-way repeated measures ANOVAs for IL-6 and IL-10 cytokines were also used to compare stimulated and unstimulated cytokine concentrations for each FM participant pre- and post-treatment or placebo interventions. The Fisher’s LSD post-hoc test was used as two separate group means can be directly compared to each other. The Pearson correlation coefficient was used to determine the strength of the linear relationship between IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention for each FM participant.

Additional analyses were done for released IL-6 and IL-10 cytokines from stimulated and unstimulated PBMCs for female and male HCs. Separate one-tailed Student’s t-tests for IL-6 and IL-10 cytokines were used to compare the effective change in stimulated and unstimulated cytokine concentrations from female and male HCs. Separate one-tailed Student’s t-tests for IL-6 and IL-10 cytokines were also used to compare stimulated and unstimulated cytokine concentrations from pre-intervention FM participants and HCs. Data was presented as means ± standard error of the mean (SEM). A p-value of less than or equal to 0.05 was significant.
Chapter 4. Results

4.1 Demographic Data

Fibromyalgia (FM) can occur at any age, but it is most commonly seen in middle aged individuals. Demographic information was collected from FM participants in both the treatment and placebo groups, and healthy controls (HCs). The sex and age of FM participants and HCs were recorded, along with the height and mass of FM participants, as shown in Table 4.

Table 4. Demographic information for fibromyalgia (FM) participants in the treatment (n=7) and placebo (n=6) groups and healthy controls (HCs; n=10).

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Mass (kg)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCs</td>
<td>27 ± 2.1;</td>
<td>N/A</td>
<td>N/A</td>
<td>7 Females;</td>
</tr>
<tr>
<td></td>
<td>48 ± 3.8</td>
<td></td>
<td></td>
<td>3 Males</td>
</tr>
<tr>
<td>FM Treatment</td>
<td>53.3 ± 2.4</td>
<td>162.4 ± 2.2</td>
<td>79.7 ± 7.7</td>
<td>7 Females</td>
</tr>
<tr>
<td>FM Placebo</td>
<td>51.5 ± 2.1</td>
<td>160.8 ± 2.2</td>
<td>97.7 ± 14.7</td>
<td>6 Females</td>
</tr>
</tbody>
</table>

4.2 HC Released Cytokine Concentration Sex Differences

4.2.1 HC Interleukin-6 (IL-6) Concentrations Released from Stimulated and Unstimulated Peripheral Blood Mononuclear Cells (PBMCs)

There was higher released IL-6 concentrations from stimulated PBMCs in HC males (n=3) compared to females (n=7), as shown in Figure 2a (p=0.0013). There was no significant difference in released IL-6 concentrations from unstimulated PBMCs in HC males or females, as shown in Figure 2b (p=0.25). These data suggest potential sex differences for the release of IL-6 from stimulated PBMCs.
Figure 2. Interleukin-6 (IL-6) concentration release from healthy control (HC) stimulated and unstimulated peripheral blood mononuclear cells (PBMCs).

PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-6 concentrations were determined in pg/mL for male (n=3) and female (n=7) HCs. a) Stimulated PBMCs from HC males had a higher IL-6 concentration of 89154.7 ± 24427 pg/mL, compared to HC females with an IL-6 concentration of 24421.5 ± 2504.6 pg/mL (p=0.0013). b) Unstimulated PBMCs from HC males had an IL-6 concentration of 9.3 ± 1.1 pg/mL, compared to HC females with an IL-6 concentration of 13.5 ± 3.7 pg/mL (p=0.25). Data are presented as mean ± standard error of the mean (SEM). * represents a significant difference (p ≤ 0.05) between males and females.
4.2.2 HC Interleukin-10 (IL-10) Concentrations Released from Stimulated and Unstimulated PBMCs

There was higher released IL-10 cytokine concentrations from stimulated PBMCs in HC males (n=3) compared to females (n=6), as shown in Figure 3a (p=0.042). There was also higher released IL-10 cytokine concentrations from unstimulated PBMCs in HC males compared to females, as shown in Figure 3b (p=0.037). These data suggest potential sex differences for the release of IL-10 from stimulated and unstimulated PBMCs.
Interleukin-10 (IL-10) concentration release from healthy control (HC) stimulated and unstimulated peripheral blood mononuclear cells (PBMCs).

PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-10 concentrations were determined in pg/mL for male (n=3) and female (n=6) HCs. a) Stimulated PBMCs from HC males had a higher IL-10 concentration of 3710.8 ± 1255.6 pg/mL, compared to HC females with an IL-10 concentration of 1555.8 ± 470.3 pg/mL (p=0.042). b) Unstimulated PBMCs from HC males had a higher IL-10 concentration of 4.3 ± 0.4 pg/mL, compared to HC females with an IL-10 concentration of 3.7 ± 0.08 pg/mL (p=0.037). Data are presented as mean ± standard error of the mean (SEM). * represents a significant difference (p ≤ 0.05) between males and females.
4.2.3 HC Effective Change Ratios in Released IL-6 Concentrations

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-6 released from PBMCs for female (n=7) and male (n=3) HCs were determined, as shown in Figure 4. A significant difference in IL-6 effective change ratios was observed (p=0.00058) between males and females. These data suggest potential sex differences for the release of IL-6 from stimulated and unstimulated PBMCs.

Figure 4. The effective change ratios in interleukin-6 (IL-6) for female and male healthy controls (HCs).

The stimulated to unstimulated cytokine concentration ratios for IL-6 released from peripheral blood mononuclear cells (PBMCs) was determined for female (n=7) and male (n=3) HCs. The effective change ratios in IL-6 were 2639.1 ± 548.6-fold for females, and 9359.7 ± 1762.9-fold for males (p=0.00058). Data are presented as mean ± standard error of the mean (SEM). * represents a significant difference (p ≤ 0.05) between males and females.

4.2.3 HC Effective Change Ratios in Released IL-10 Concentrations

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-10 released from PBMCs for female (n=6) and male (n=3) HCs were determined, as shown in Figure 5. No significant difference in IL-10 effective change ratios was observed (p=0.086)
between males and females. These data do not suggest potential sex differences for the release of IL-10 from stimulated and unstimulated PBMCs.

Figure 5. The effective change ratios in interleukin-10 (IL-10) for female and male healthy controls (HCs). The stimulated to unstimulated cytokine concentration ratios for IL-10 released from peripheral blood mononuclear cells (PBMCs) was determined for female (n=6) and male (n=3) HCs. The effective change ratios in IL-10 were $431.6 \pm 141.0$-fold for females, and $887.0 \pm 330.2$-fold for males ($p=0.086$). Data are presented as mean ± standard error of the mean (SEM).

4.3 Cytokine Concentration for Pre-Intervention FM Participants and HCs

4.3.1 IL-6 Concentrations Released from Stimulated and Unstimulated PBMCs

Since all FM participants enrolled in the study were female, only female HCs were used for data analysis to remain consistent with the majority of methods used in the literature. Released IL-6 concentrations from stimulated and unstimulated PBMCs for HCs (n=7) and FM participants in both treatment and placebo groups pre-intervention (n=13) were determined, as shown in Figures 6a and 6b, respectively.
Figure 6. Interleukin-6 (IL-6) concentration release from stimulated and unstimulated peripheral blood mononuclear cells (PBMCs) for fibromyalgia (FM) participants and healthy controls (HCs).

PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-6 concentrations were determined in pg/mL for FM participants in both treatment and placebo groups pre-intervention (n=13) and HCs (n=7). a) Stimulated PBMCs from FM participants had an IL-6 concentration of $30134.1 \pm 6647.4$ pg/mL, compared to HCs with an IL-6 concentration of $24421.5 \pm 2504.6$ pg/mL ($p=0.27$). b) Unstimulated PBMCs from FM participants had an IL-6 concentration of
42.9 ± 20.1 pg/mL, compared to HCs with an IL-6 concentration of 13.5 ± 3.7 pg/mL (p=0.15). Data are presented as mean ± standard error of the mean (SEM).

In Figure 6a, there was no significant difference in IL-6 concentrations released from stimulated PBMCs between HCs and FM participants (p=0.27). IL-6 concentrations of 2442.5 ± 2504.6 pg/mL and 30134.1 ± 6647.4 pg/mL were observed for HCs and FM participants from stimulated PBMCs, respectively. There was also no significant difference in IL-6 concentrations released from unstimulated PBMCs between HCs and FM participants (p=0.15), as shown in Figure 6b. IL-6 concentrations of 13.5 ± 3.7 pg/mL and 42.9 ± 20.1 pg/mL were observed for HCs and FM participants from unstimulated PBMCs, respectively. Although there were no significant differences between the HC and FM participant groups, there was a non-significant trend where FM participants had higher IL-6 concentrations released from stimulated and unstimulated PBMCs compared to HCs, which is more pronounced by an almost 3-fold difference in the unstimulated PBMCs.

4.3.2 IL-10 Concentrations Released from Stimulated and Unstimulated PBMCs

Released IL-10 concentrations from stimulated and unstimulated PBMCs for HCs (n=6) and FM participants in both treatment and placebo groups pre-intervention (n=13) were determined, as shown in Figures 7a and 7b, respectively.
Figure 7. Interleukin-10 (IL-10) concentration release from stimulated and unstimulated peripheral blood mononuclear cells (PBMCs) for fibromyalgia (FM) participants and healthy controls (HCs).

PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-10 concentrations were determined in pg/mL for FM participants in both treatment and placebo groups pre-intervention (n=13) and HCs (n=6). a) Stimulated PBMCs from FM participants had an IL-10 concentration of 1546.7 ± 323.4 pg/mL, compared to HCs with an IL-10 concentration of 1555.8 ± 470.3 pg/mL (p=0.49). b) Unstimulated PBMCs from FM participants had an IL-10 concentration of
4.0 ± 0.2 pg/mL, compared to HCs with an IL-10 concentration of 3.7 ± 0.1 pg/mL (p=0.13). Data are presented as mean ± standard error of the mean (SEM).

In Figure 7a, there was no difference in IL-10 concentrations released from stimulated PBMCs between HCs and FM participants (p=0.49). IL-10 concentrations of 1555.8 ± 470.3 pg/mL and 1546.7 ± 323.4 pg/mL were seen for HCs and FM participants from stimulated PBMCs, respectively. There was also no difference in IL-10 concentrations released from unstimulated PBMCs between HCs and FM participants (p=0.13), as shown in Figure 7b. IL-10 concentrations of 3.7 ± 0.1 pg/mL and 4.0 ± 0.2 pg/mL were observed for HCs and FM participants from unstimulated PBMCs, respectively.

4.3.3 Effective Change Ratios in Released IL-6 Concentrations

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-6 released from PBMCs for HCs (n=7) and FM participants in both treatment and placebo groups pre-intervention (n=13) were determined, as shown in Figure 8.
Figure 8. The effective change ratios in interleukin-6 (IL-6) for fibromyalgia (FM) participants and healthy controls (HCs).

The stimulated to unstimulated cytokine concentration ratios for IL-6 released from peripheral blood mononuclear cells (PBMCs) was determined for HCs (n=7) and FM participants in both treatment and placebo groups pre-intervention (n=13). The effective change ratios in IL-6 were $2639.1 \pm 548.6$-fold for HCs, and $2363.7 \pm 693.6$-fold for FM participants (p=0.40). Data are presented as mean ± standard error of the mean (SEM).

In Figure 8, no significant difference in IL-6 effective change ratios was observed between HCs and FM participants (p=0.40). There were IL-6 effective change ratios of $2639.1 \pm 548.6$-fold and $2363.7 \pm 693.6$-fold for HCs and FM participants, respectively. Although there was no significant difference between the HC and FM participant groups, there was a non-significant trend where FM participants had a lower IL-6 effective change ratio compared to HCs.

**4.3.4 Effective Change Ratios in Released IL-10 Concentrations**

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-10 released from PBMCs for HCs (n=6) and FM participants in both treatment and placebo groups pre-intervention (n=13) were determined, as shown in Figure 9.
The stimulated to unstimulated cytokine concentration ratios for IL-10 released from peripheral blood mononuclear cells (PBMCs) was determined for HCs (n=6) and FM participants in both treatment and placebo groups pre-intervention (n=13). The effective change ratios in IL-10 were 431.6 ± 141-fold for HCs, and 381.4 ± 75.7-fold for FM participants (p=0.37). Data are presented as mean ± standard error of the mean (SEM).

In Figure 9, no significant difference in IL-10 effective change ratios was observed between HCs and FM participants (p=0.37). There were IL-10 effective change ratios of 431.6 ± 141-fold and 381.4 ± 75.7-fold for HCs and FM participants, respectively. Although there was no significant difference between the HC and FM participant groups, there was a non-significant trend where FM participants had a lower effective change ratio compared to HCs.

4.4 Cytokine Concentration for FM Participants in Treatment and Placebo Groups

4.4.1 IL-6 Concentrations Released from Stimulated PBMCs Pre- and Post-Interventions

Repeated measures for released IL-6 concentrations from stimulated PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-interventions were determined, as shown in Figure 10.
Figure 10. Repeated measures for interleukin-6 (IL-6) from stimulated peripheral blood mononuclear cells (PBMCs) from fibromyalgia (FM) participants pre- and post-interventions. PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-6 concentrations were determined in pg/mL for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-intervention. FM participants in the treatment group had pre- and post-intervention IL-6 concentrations of 23625 ± 9571 pg/mL and 42767 ± 9571 pg/mL (p=0.36), compared to the placebo group which had pre- and post-intervention IL-6 concentrations of 37728 ± 17479 pg/mL and 72685 ± 17479 pg/mL (p=0.13), respectively. There was a significant group effect between the treatment and placebo groups (p=0.016) and no significant difference between any groups (p=0.60). Data are presented as mean ± standard error of the mean (SEM).

In Figure 10, there was no significant difference in IL-6 concentrations released from stimulated PBMCs between FM participants in the treatment and placebo groups pre- and post-interventions (p=0.60). FM participants in the treatment group had pre- and post-intervention IL-6 concentrations of 23625 ± 9571 pg/mL and 42767 ± 9571 pg/mL (p=0.36), compared to the placebo group which had pre- and post-intervention IL-6 concentrations of 37728 ± 17479 pg/mL and 72685 ± 17479 pg/mL (p=0.13). There was a significant group effect between the treatment and placebo groups (p=0.016).
4.4.2 IL-6 Concentrations Released from Unstimulated PBMCs Pre- and Post-Interventions

Repeated measures for the released IL-6 concentrations from unstimulated PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-interventions were determined, as shown in Figure 11.

Figure 11. Repeated measures for interleukin-6 (IL-6) from unstimulated peripheral blood mononuclear cells (PBMCs) from fibromyalgia (FM) participants pre- and post-interventions. IL-6 concentrations were determined in pg/mL for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-intervention. FM participants in the treatment group had pre- and post-intervention IL-6 concentrations of 29 ± 26.5 pg/mL and 81.9 ± 26.5 pg/mL (p=0.42), compared to the placebo group which had pre- and post-intervention IL-6 concentrations of 59.2 ± 5.1 pg/mL and 69.3 ± 5.1 pg/mL (p=0.88), respectively. There was no group effect between the treatment and placebo groups (p=0.83) and no significant difference between any groups (p=0.65). Data are presented as mean ± standard error of the mean (SEM).

In Figure 11, there was no significant difference in IL-6 concentrations released from unstimulated PBMCs between FM participants in the treatment and placebo groups pre- and post-interventions (p=0.65). FM participants in the treatment group had pre-and post-intervention IL-6 concentrations of 29 ± 26.5 pg/mL and 81.9 ± 26.5 pg/mL (p=0.42), compared to the
placebo group which had pre- and post-intervention IL-6 concentrations of 59.2 ± 5.1 pg/mL and 69.3 ± 5.1 pg/mL (p=0.88), respectively. There was no group effect between the treatment and placebo groups (p=0.83).

4.4.3 IL-10 Concentrations Released from Stimulated PBMCs Pre- and Post-Interventions

Repeated measures for the released IL-10 concentrations from stimulated PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-interventions were determined, as shown in Figure 12.

![Figure 12](image)

Figure 12. Repeated measures for interleukin-10 (IL-10) from stimulated peripheral blood mononuclear cells (PBMCs) from fibromyalgia (FM) participants pre- and post-interventions. PBMCs were stimulated with phytohemagglutinin-P (PHA-P). IL-10 concentrations were determined in pg/mL for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-intervention. FM participants in the treatment group had pre- and post-intervention IL-10 concentrations of 1503 ± 377.3 pg/mL and 2257 ± 377.3 pg/mL (p=0.49), compared to the placebo group which had pre- and post-intervention IL-10 concentrations of 1598 ± 825.8 pg/mL and 3250 ± 825.8 pg/mL (p=0.17), respectively. There was no group effect between the treatment and placebo groups (p=0.20) and no significant difference between any groups (p=0.57). Data are presented as mean ± standard error of the mean (SEM).
In Figure 12, there was no significant difference in IL-10 concentrations released from stimulated PBMCs between FM participants in the treatment and placebo groups pre- and post-interventions (p=0.57). FM participants in the treatment group had pre- and post-intervention IL-10 concentrations of 1503 ± 377.3 pg/mL and 2257 ± 377.3 pg/mL (p=0.49), compared to the placebo group which had pre- and post-intervention IL-10 concentrations of 1598 ± 825.8 pg/mL and 3250 ± 825.8 pg/mL (p=0.17), respectively. There was no group effect between the treatment and placebo groups (p=0.20).

4.4.4 IL-10 Concentrations Released from Unstimulated PBMCs Pre- and Post-Interventions

Repeated measures for the released IL-10 concentrations from unstimulated PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-interventions were determined, as shown in Figure 13.
Figure 13. Repeated measures for interleukin-10 (IL-10) from unstimulated peripheral blood mononuclear cells (PBMCs) from fibromyalgia (FM) participants pre- and post-interventions. IL-10 concentrations were determined in pg/mL for FM participants in the treatment (n=7) and placebo (n=6) groups pre- and post-intervention. FM participants in the treatment group had pre- and post-intervention IL-10 concentrations of 4.2 ± 0.3 pg/mL and 4.9 ± 0.3 pg/mL (p=0.59), compared to the placebo group which had pre- and post-intervention IL-10 concentrations of 3.7 ± 1.2 pg/mL to 6.1 ± 1.2 pg/mL (p=0.07). There was no group effect between the treatment and placebo groups (p=0.62) and no significant difference between any groups (p=0.30). Data are presented as mean ± standard error of the mean (SEM).

In Figure 13, there was no significant difference in IL-10 concentrations released from unstimulated PBMCs between FM participants in the treatment and placebo groups pre- and post-interventions (p=0.30). FM participants in the treatment group had pre- and post-intervention IL-10 concentrations of 4.2 ± 0.3 pg/mL and 4.9 ± 0.3 pg/mL (p=0.59), compared to the placebo group which had pre- and post-intervention IL-10 concentrations of 3.7 ± 1.2 pg/mL and 6.1 ± 1.2 pg/mL (p=0.07), respectively. There was no group effect between the treatment and placebo groups (p=0.62).
4.4.5 Effective Change Ratios in Released IL-6 Concentrations Pre- and Post-Interventions

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-6 released from PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups for pre- and post-interventions were determined, as shown in Figures 14a and 14b.
Figure 14. The effective change ratios in interleukin-6 (IL-6) for pre- and post-intervention fibromyalgia (FM) participants.

a) The stimulated to unstimulated cytokine concentration ratios for IL-6 released from peripheral blood mononuclear cells (PBMCs) were determined for FM participants pre- and post-interventions as indicated by the white and light grey bars, respectively, for the treatment (n=7) and placebo (n=6) groups, as well as for healthy controls (HCs; n=7) as indicated by the dashed black horizontal line. The effective change ratios in IL-6 for pre- and post-treatment FM participants were 2718.8 ± 1037.1-fold and 4289.4 ± 1977.1-fold, compared to pre- and post-placebo FM participants which was 1949.5 ± 965.4-fold and 2459.4 ± 1008.8-fold, respectively. There were no significant differences from pre- to post-interventions for the treatment (p=0.43) or placebo (p=0.81) groups, and there was no overall significant difference between groups.
b) The repeated measures effective change ratios in IL-6 from pre- to post-intervention in the treatment and placebo groups for each FM participant. Data are presented as mean ± standard error of the mean (SEM).

In Figure 14a, no significant difference in IL-6 effective change ratios was observed between FM participants in the treatment and placebo groups for pre- and post-interventions (p=0.71). The effective change ratios in IL-6 for pre- and post-treatment FM participants were 2718.8 ± 1037.1-fold and 4289.4 ± 1977.1-fold, compared to pre- and post-placebo FM participants which were 1949.5 ± 965.4-fold and 2459.4 ± 1008.8-fold, respectively. There was no significant difference between FM participants in the treatment (p=0.43) and placebo (p=0.81) groups pre- and post-intervention. Figure 14b shows the variation between FM participants for the effective change ratios in IL-6 from pre- to post-intervention in the treatment and placebo groups. In the treatment group, four FM participants had higher IL-6 effective change ratios post-intervention compared to pre-intervention, and three FM participants had lower IL-6 effective change ratios post-intervention compared to pre-intervention. In the placebo group, four FM participants had higher IL-6 effective change ratios post-intervention compared to pre-intervention, and two FM participants had lower IL-6 effective change ratios post-intervention compared to pre-intervention.

4.4.6 Effective Change Ratios in Released IL-10 Concentrations Pre- and Post-Interventions

The effective change in stimulated to unstimulated cytokine concentration ratios for IL-10 released from PBMCs for FM participants in the treatment (n=7) and placebo (n=6) groups for pre- and post-interventions were determined, as shown in Figures 15a and 15b.
Figure 15. The effective change ratios in interleukin-10 (IL-10) for pre- and post-intervention fibromyalgia (FM) participants.

a) The stimulated to unstimulated cytokine concentration ratios for IL-10 released from peripheral blood mononuclear cells (PBMCs) were determined for FM participants pre- and post-interventions as indicated by the white and dark grey bars, respectively, for the treatment (n=7) and placebo (n=6) groups, as well as for healthy controls (HCs; n=6) as indicated by the dashed black horizontal line. The effective change ratios in IL-10 for pre- and post-treatment FM participants were 337.3 ± 108.7-fold and 511.3 ± 150-fold, compared to pre- and post-placebo FM participants which were 423.9 ± 110.9-fold and 537.7 ± 124.9-fold, respectively. There were no significant differences from pre- to post-interventions for the treatment (p=0.41) or placebo
(p=0.64) groups, and there was no overall significant difference between groups (p=0.82). b) The repeated measures effective changes ratios in IL-10 from pre- to post-intervention in the treatment and placebo groups for each FM participant. Data are presented as mean ± standard error of the mean (SEM).

In Figure 15a, no significant difference in IL-10 effective change ratios was observed between FM participants in the treatment and placebo groups for pre- and post-interventions (p=0.82). The effective change ratios in IL-10 for pre- and post-treatment FM participants were 337.3 ± 108.7-fold and 511.3 ± 150-fold, compared to pre- and post-placebo FM participants which were 423.9 ± 110.9-fold and 537.7 ± 124.9-fold, respectively. There were no significant differences between FM participants in the treatment (p=0.41) and placebo (p=0.64) groups pre- and post-intervention. Figure 15b shows the variation between FM participants for the effective change ratios in IL-10 from pre- to post-intervention in the treatment and placebo groups. In the treatment group, five FM participants had higher IL-10 effective change ratios post-intervention compared to pre-intervention, and two FM participants had lower IL-10 effective change ratios post-intervention compared to pre-intervention. In the placebo group, two FM participants had higher IL-10 effective change ratios post-intervention compared to pre-intervention, and four FM participants had lower IL-10 effective change ratios post-intervention compared to pre-intervention.

4.4.7 IL-6 and IL-10 Delta Effective Change Ratios from Pre- to Post-Interventions

The correlation of IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention for each FM participant (n=13) were determined, as shown in Figures 16.
Figure 16. The correlation of interleukin-6 (IL-6) and interleukin-10 (IL-10) delta effective change ratios for each fibromyalgia (FM) participant. The IL-6 and IL-10 delta effective change ratios were determined from the difference in effective change ratios pre- to post-intervention for each FM participant (n=13). A positive correlation was observed (y=0.07460x+61.67), which was also significant (p=0.0073).

In Figure 16, a significant positive correlation was observed between IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention for each FM participant (p=0.0073). Six FM participants with positive IL-6 delta effective change ratios also had positive IL-10 delta effective change ratios, while four FM participants with negative IL-6 delta effective change ratios also had negative IL-10 delta effective change ratios. Three FM participants had opposite IL-6 and IL-10 delta effective change ratios.
Chapter 5. Discussion

There is currently no accurate diagnostic method or definitive treatment approach for fibromyalgia (FM). Behm et al. 2012 evaluated the immune system function of individuals with FM compared to healthy controls (HCs) by quantifying cytokines released from stimulated peripheral blood mononuclear cells (PBMCs), and suggested the potential of this method to be used as a FM diagnostic approach. Cytokines also play a role in various comorbidities and symptoms associated with FM, including hyperalgesia, allodynia, cognitive dysfunction and fatigue, which supports their analysis in FM research. In the present study, the capacity of unstimulated PBMCs, and PBMCs stimulated with the mitogen phytohemagglutinin-P (PHA-P) to release interleukin-6 (IL-6) and interleukin-10 (IL-10) cytokines was investigated in individuals with FM to determine if their cytokine concentrations were lower compared to HCs. IL-6 and IL-10 cytokines from stimulated PBMCs were analyzed as their concentrations have been observed in previous research to be lower in individuals with FM. IL-6 and IL-10 cytokines from unstimulated PBMCs of individuals with FM were also analyzed to determine if these cells were isolated with an elevated activation state, causing the release of cytokines. Cytokines released from stimulated and unstimulated PBMCs were used instead of basal circulating levels in the blood as the immune system function of FM participants was investigated. Analyzing cytokines released from mitogen-stimulated and unstimulated immune cells can demonstrate how cells react to immune responses in the body, and if immune cell function is altered, respectively.

The present study used the 2010 American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for FM to classify FM in participants and verify the measurement of potentially lower IL-6 and IL-10 concentrations released from stimulated FM PBMCs as previous studies by Wallace et al. 2015 and Behm et al. 2012 used this criteria and observed lower released cytokine concentrations in FM. Enzyme-linked immunosorbent assays (ELISAs) were used to determine cytokine concentrations in the present study as they have been recognized as the best method to accurately quantify IL-6 and IL-10 concentrations released from stimulated PBMCs. The ELISA method was chosen for the current study in contrast to previous research by Behm et al. 2012 and Wallace et al. 2015 who used the less widely
accepted multiplex immunoassay cytokine detection method as it has been criticized regarding its accuracy quantifying cytokine concentrations.\textsuperscript{3,4,78,79}

The effectiveness of radial shockwave therapy (RSWT) as a novel treatment method for PBMC dysfunctions in individuals with FM was also evaluated in the present study. RSWT promotes healing by producing tissue inflammation that causes cells to undergo a regenerative process, which may regulate immune cell function due to an increase in an individual’s overall well-being from reduced pain.\textsuperscript{5} Detecting higher released IL-6 and IL-10 concentrations from stimulated PBMCs before and after an individual with FM receives RSWT treatment may indicate the effectiveness of RSWT at improving PBMC function.

5.1 PBMC Function and Cytokine Release for FM Participants and HCs

In the present study, the ability of PBMCs from FM participants and HCs to be stimulated with the PHA-P mitogen and release IL-6 and IL-10 cytokines was assessed. In Figures 6a and 6b, the IL-6 concentrations released from stimulated and unstimulated PBMCs, respectively, were both non-significantly higher in FM participants compared to HCs. Of particular interest is the non-significant IL-6 concentration released from unstimulated PBMCs in FM participants that is approximately 3-fold higher compared to HCs, as shown in Figure 6b. The non-significantly higher concentration of IL-6 release from stimulated PBMCs was much less pronounced between FM participants and HCs, as shown in Figure 6a. The results from the analysis of IL-6 released from stimulated PBMCs do not agree with the literature by Wallace et al. 2015 and Behm et al. 2012 which observed lower released IL-6 concentrations from stimulated PBMCs in individuals with FM compared to HCs.\textsuperscript{3,4} The range of IL-6 concentration detected was also much lower in Behm et al. 2012, as shown in Table 1, compared to the present research.\textsuperscript{4} HCs in the present study had a mean released IL-6 concentration from unstimulated PBMCs of 13.5 ± 9.9 pg/mL, compared to 215 ± 380 pg/mL in Behm et al. 2012.\textsuperscript{4} These data were presented as mean concentration ± standard deviation (SD). HCs in the present study had a range of released IL-6 concentrations from unstimulated PBMCs of 17149.0 to 34093.8 pg/mL, compared to 1.2 to 15592 pg/mL in Behm et al. 2012.\textsuperscript{4} FM participants in the present study had a range of released IL-6 concentrations from stimulated PBMCs of 32.3 to 85140.8 pg/mL, compared to 3.1 to 2255 pg/mL in Behm et al. 2012.\textsuperscript{4} These discrepancies in FM participant IL-6
ranges could be due to differences in FM participant characteristics, and screening and inclusion criteria between the previous and present studies, as shown in Table 5.³⁴

Table 5. Differences in FM participant characteristics, and screening and inclusion criteria between the present study and studies by Behm et al. 2012 and Wallace et al. 2015. For each study, the total number of male and female FM participants, the FM identification criteria used to include FM participants, mean age in years, FM medication use during the study, and method used to quantify cytokine concentrations were examined.

<table>
<thead>
<tr>
<th>Study</th>
<th>Total FM Participants</th>
<th>FM Identification</th>
<th>Age</th>
<th>FM Medication</th>
<th>Method for Cytokine Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present Study</td>
<td>13 female</td>
<td>ACR 2010</td>
<td>52.5</td>
<td>Used during study</td>
<td>ELISA</td>
</tr>
<tr>
<td>Behm et al. 2012</td>
<td>98 female, 12 male</td>
<td>FM diagnosis &gt;1 year, ACR 1990 and 2010</td>
<td>52.2</td>
<td>Stopped use two weeks before beginning study</td>
<td>Luminex xMAP™ multiplex immunoassay bead array</td>
</tr>
<tr>
<td>Wallace et al. 2015</td>
<td>98 female, 12 male</td>
<td>FM diagnosis &gt;1 year, ACR 1990 and 2010</td>
<td>52.2</td>
<td>Stopped use two weeks before beginning study</td>
<td>Luminex multiplex immunoassay bead</td>
</tr>
</tbody>
</table>

Abbreviations: ACR – American College of Rheumatology Preliminary Diagnostic Criteria for FM, ELISA – Enzyme Linked Immunosorbent Assay, FM – Fibromyalgia, SEM – Standard Error of the Mean

The released IL-6 range results from stimulated PBMCs in the present study are more inline with results from the study by Macedo et al. 2007 who observed average released IL-6 concentrations from stimulated PBMCs of 33223.19 ± 4155.03 pg/mL, and 33884.14 ± 3626.81 pg/mL for FM participants and HCs, respectively.⁷⁶ These data were presented as mean concentration ± standard error.⁷⁶ Although the data from Macedo et al. 2007 support lower released IL-6 concentrations from stimulated PBMCs in FM participants compared to controls similar to Behm et al. 2012, their use of an automated biochip array analyzer was able to quantify more comparable released IL-6 concentrations to the present research.⁴,⁷⁶ The higher IL-6 concentrations from unstimulated PBMCs in FM participants in the present study, although not
significant, could indicate a potential immunological dysfunction of immune cells. This dysfunction could have caused PBMCs from individuals with FM to be isolated with an elevated activation state, which could have caused a higher IL-6 cytokine release in unstimulated FM PBMCs. It is known that IL-6 is involved in pain perception in an individual, and altered PBMC function in FM participants could have potentially caused higher amounts of IL-6 to be released \textit{in vivo} following PBMC activation by an immune system response. This response could have possibly contributed to increased pain levels related to FM.

To account for the effects of IL-6 released by unstimulated PBMCs, the effective change ratios in released IL-6 concentration from stimulated and unstimulated PBMCs were determined for FM participants and HCs, as shown in Figure 8. There was no significant difference in the IL-6 effective change ratios between FM participants and HCs. No significant difference for the IL-6 effective change ratios between FM participants and HCs could be partly due to the influence of IL-6 concentrations from unstimulated PBMCs as the effective change ratios are dependent on this denominator.

In addition to analyzing the release of IL-6 from stimulated and unstimulated PBMCs from FM participants and HCs, the release of IL-10 was also investigated. Figures 7a and 7b demonstrate there was no difference in the concentrations of IL-10 released from stimulated or unstimulated PBMCs, respectively. Unlike IL-6 concentrations released from stimulated and unstimulated PBMCs, there was no trend for elevated IL-10 concentrations from stimulated or unstimulated PBMCs in FM participants compared to controls. In contrast to results from the present study, Macedo et al. 2007 observed higher released IL-10 concentrations from stimulated PBMCs in FM participants compared to controls. It is known that IL-10 has anti-inflammatory properties, and lower released IL-10 concentrations in FM participant stimulated PBMCs could have indicated a blunted anti-inflammatory response, which could have also affected released IL-6 concentrations.

To account for the effects of IL-10 released by unstimulated PBMCs, the effective change ratios in IL-10 concentration released from stimulated and unstimulated PBMCs were determined for FM participants and HCs, as shown in Figure 9. There was no significant difference in the IL-10 effective changes between FM participants and HCs. It is important to note that all the differences in IL-10 concentrations and effective changes observed between FM participants and HCs were not significant.
In the present study, the stimulated PBMC release of IL-6 and IL-10 is not significantly different between FM participants and HCs, which does not suggest altered PBMC function in individuals with FM. Other studies have postulated that altered PBMC function in individuals with FM potentially affects interactions of the immune system with other systems of the body, such as the nervous system, as cytokines are responsible for communication between cells. An immune system dysfunction in individuals with FM could have affected proper healing and repair of tissues, including neural tissues, which could have led to chronic pain. The contrast between the present research and previous research studies by Wallace et al. 2015 and Behm et al. 2012 indicating individuals with FM had a blunted response in cytokine release from stimulated PBMCs could be due to differences in screening and inclusion criteria used, as shown in Table 5, along with the approximately 9-times higher FM participant cohort sizes from both previous research studies compared to the present study. Overall, the present study determined that quantifying IL-6 and IL-10 concentrations from stimulated PBMCs, or observing effective change ratios are not effective at distinguishing FM participants from HCs due to non-significant differences observed in IL-6 and IL-10 concentrations released from stimulated PBMCs. Although Behm et al. 2012 and Wallace et al. 2015 obtained significant results without determining the homogeneity of their FM participants or identifying FM subgroups, based on the highly variable data in the present study, a larger FM participant pool with identifiable FM subgroups in future research could produce significant results regarding differences in cytokine release from stimulated PBMCs. The minimum sample size of FM participants needed in future studies to obtain significance if it exists would be determined using an a priori power analysis. There is also heterogeneity within the HC population which should be minimized in future research by including more factors in the inclusion and exclusion criteria, including height, mass, and smoking history of HCs. Due to a lack of a definitive FM diagnosis method, the group of FM participants in the present study could have potentially been highly heterogeneous with respect to the large variations in the data observed between FM participants, which could have resulted in no significant differences being observed between FM participants and HCs. No significantly observed differences for cytokine release between individuals with FM and HCs in the present study indicates certain parameters should be accounted for in future research, including increasing the FM sample size, and determining the presence of depression, symptom severity, body-mass index, and homogeneity of FM participants. These parameters could better define the
FM population studied, and determine if cytokines could be a potentially effective biomarker for diagnosing FM.

5.2 Effectiveness of RSWT at Improving PBMC Function and Cytokine Release

In the present study, the ability of RSWT to improve PBMC function in FM participants pre- and post-treatment was assessed. In Figures 10 and 12, there were no significant differences for the concentrations of IL-6 and IL-10 released from stimulated PBMCs in FM participants from the treatment and placebo groups pre- and post-interventions. There was a non-significant trend for the treatment and placebo groups from Figures 10 and 12 to have higher stimulated PBMC released IL-6 or IL-10 concentrations pre- to post-interventions. The treatment and placebo groups in Figure 10 had p values for the stimulated PBMC release of IL-6 pre- to post-interventions of 0.36 and 0.13, respectively, while the treatment and placebo groups in Figure 12 had p values for the stimulated PBMC release of IL-10 pre- to post-interventions of 0.49 and 0.17, respectively. These higher IL-6 or IL-10 concentrations released from stimulated PBMCs in the treatment and placebo groups could potentially be due to placebo effects. For the treatment group, this potential placebo effect is where FM participants believe their condition is improving due to the RSWT treatments, but any changes they experience are not from the actual treatment. For the placebo group, this potential placebo effect is where FM participants believe they are receiving the RSWT treatments and their condition is improving, but they are actually not receiving the treatment. In Figure 10, there was also a significant difference between treatment and placebo groups when the pre- and post-interventions were combined, where released IL-6 concentrations were lower in the treatment group compared to the placebo group. This finding could also be due to placebo effects.

In addition to quantifying released IL-6 and IL-10 concentrations from stimulated PBMCs, concentrations of IL-6 and IL-10 released from unstimulated PBMCs of FM participants in Figures 11 and 13 yielded non-significant differences between the treatment and placebo groups from pre- to post-interventions. There were no trends observed for the treatment and placebo groups from Figures 11 and 13 to have higher unstimulated PBMC released IL-6 or IL-10 concentrations pre- to post-interventions. The treatment and placebo groups in Figure 11 had p values for the unstimulated PBMC release of IL-6 pre- to post-interventions of 0.42 and 0.88, respectively. The treatment and placebo groups in Figure 13 had p values for the
unstimulated PBMC release of IL-10 pre- to post-interventions of 0.59 and 0.07, respectively. There was a non-significant trend for the placebo group from Figure 13 to have higher unstimulated PBMC released IL-10 concentrations post- compared to pre-interventions.

In the present study, the unstimulated PBMC release of IL-6 and IL-10 concentrations was not significantly different between FM participants in the treatment and placebo groups pre- to post-interventions. The lack of significant differences between the treatment and placebo groups pre- to post-intervention indicated that RSWT did not significantly alter PBMC function.

To account for the effects of IL-6 released by unstimulated PBMCs, the effective change ratios in IL-6 concentration released from stimulated and unstimulated PBMCs were determined for FM participants in treatment and placebo groups pre- and post-interventions, as shown in Figures 14a and 14b. The effective change ratio in IL-6 concentration for HCs was also included for comparisons. In Figure 14a, there was no significant difference between FM participants in the treatment (p=0.43) and placebo (p=0.81) groups pre- and post-intervention. There was insufficient evidence to support the hypothesis of RSWT treatment promoting local healing and tissue repair that would cause a reduction in pain and increase the FM participant’s overall well-being, leading to improved PBMC function and cytokine release following stimulation. In Figure 14b, some individual FM participants in the treatment and placebo groups demonstrated a lower IL-6 effective change ratio post-intervention compared to pre-intervention. In the treatment group, there is considerable variation in IL-6 effective change ratios from pre- to post-intervention. There are three FM participants who had lower IL-6 effective change ratios post- compared to pre-treatment, and four FM participants who had higher IL-6 effective change ratios post- compared to pre-treatment. In the placebo group, there was also considerable variability in IL-6 effective change ratio from pre- to post-intervention. There were two FM participants who had lower IL-6 effective change ratios post- compared to pre-placebo, and four FM participants who had higher IL-6 effective change ratios post- compared to pre-placebo. This variability in IL-6 effective change ratios between FM participants pre- to post-intervention could indicate more than one FM subgroup is present, where RSWT is more effective at treating one group compared to others.²⁶,²⁷ Future research could study individuals with FM who have lower or higher IL-6 effective change ratios pre- to post-intervention separately to determine if they could be certain FM subgroups. It is important to note that all differences in IL-6 concentrations seen
between FM participants in the placebo and treatment groups pre- and post-intervention were not significant.

To account for the effects of IL-10 released by unstimulated PBMCs, the effective change ratios in IL-10 concentration released from stimulated and unstimulated PBMCs were determined for FM participants in treatment and placebo groups pre- and post-interventions, as shown in Figures 15a and 15b. The effective change ratio in IL-10 concentration for HCs was also included for comparisons. In Figure 15a, there were no significant differences between FM participants in the treatment (p=0.41) and placebo (p=0.64) groups pre- and post-intervention. There was insufficient evidence to support the hypothesis of RSWT treatment promoting local healing and tissue repair that would result in a reduction in pain and increase the FM participant’s overall well-being, leading to improved PBMC function and cytokine release following stimulation. In Figure 15b, some individual FM participants in the treatment and placebo groups demonstrated a lower IL-10 effective change ratio post-intervention compared to pre-intervention. In the treatment group, there was considerable variability in IL-10 effective change ratios from pre- to post-intervention. There were two FM participants who had lower IL-10 effective change ratios post- compared to pre-treatment, and five FM participants who had higher IL-10 effective change ratios post- compared to pre-treatment. In the placebo group, there was also considerable variability in IL-10 effective change ratio from pre- to post-intervention. There were four FM participants who had lower IL-10 effective change ratios post- compared to pre-placebo, and two FM participants who had higher IL-10 effective change ratios post-compared to pre-placebo. This variability in IL-10 effective change ratios between FM participants pre- to post-intervention could indicate more than one FM subgroup is present, where RSWT is more effective at treating one group compared to others.26,27 Future research could study individuals with FM who have lower or higher IL-10 effective change ratios pre- to post-intervention separately to determine if they could be certain FM subgroups. It is important to note that all differences in IL-10 concentrations seen between FM participants in the placebo and treatment groups pre- and post-intervention were not significant.

A correlation between IL-6 and IL-10 delta effective change ratios from the difference in effective change ratios pre- to post-intervention for each FM participant in the treatment and placebo groups was determined, as shown in Figure 16. A significant positive correlation was observed between the IL-6 and IL-10 delta effective change ratios for each participant.
Out of the 13 total FM participants, six FM participants with positive IL-6 delta effective change ratios also had positive IL-10 delta effective change ratios, while four FM participants with negative IL-6 delta effective change ratios also had negative IL-10 delta effective change ratios. Three FM participants had opposite IL-6 and IL-10 delta effective change ratios. The evidence of a significant positive correlation of IL-6 and IL-10 delta effective change ratios observed in the present study could indicate that IL-6 and IL-10 are correlated with each other in FM participants. Bazzichi et al. 2007 suggested that IL-6 and IL-10 plasma concentration increases were correlated with each other due to the role of IL-10 as an anti-inflammatory cytokine to balance increases of the pro-inflammatory cytokine IL-6. Future research could analyze the correlation of IL-6 and IL-10 cytokines further to determine if they could be used to identify specific FM subgroups.

In addition to their importance in the immune system, cytokines are also important in the nervous system. In the body, the immune system can be activated from the stimulation of immune cells after experiencing trauma, infection or inflammation, and this process involves the nervous system which results in the production of inflammatory cytokines. The interactions between the nervous and immune systems in individuals with FM could be affected due to the presence of aberrant cytokine concentrations as these are common molecules for communication between cells. Since FM chronic pain is not from a physical injury, it is suggested that deregulations in the nervous system are causing an individual with FM to feel pain in areas where no physical damage is seen. In this case, neural pain pathway dysfunctions cause improper pain signals to be sent to the brain and an individual to think they are experiencing pain. With the knowledge that cytokines are used for communication between the nervous and immune systems, the improvement of PBMC function and cytokine release from a reduction in pain and increase in overall well-being can be suggested. Although significant differences were not seen for higher IL-6 or IL-10 concentrations from stimulated PBMCs following RSWT treatment, a larger sample size and stricter screening and inclusion criteria could be used in future research to determine if significant differences are seen in a specific FM subgroup. These additions to future research could determine if RSWT is a potentially effective treatment approach for FM. Overall, based on the results from the present study, RSWT did not have an effect on increasing the release of IL-6 and IL-10 cytokines from pre- to post-interventions following treatment.
5.3 IL-6 and IL-10 Cytokine Analyses Considerations

For the analyses done on released IL-6 and IL-10 concentrations from stimulated PBMCs, there are some considerations that need to be addressed. While studies by Behm et al. 2012 and Wallace et al. 2015 analyzing isolated PBMCs stimulated with a mitogen assessed immune system function in vitro, environmental influences from other systems in the body were absent, which could greatly alter cytokine release. Since PHA-P can only stimulate a T-cell response from isolated PBMCs, potentially different results could be obtained if a different compound was used that stimulated PBMCs and monocytes. In the in vitro analysis of isolated PBMCs, unstimulated PBMCs should not release cytokines; however, they may become activated and release some cytokines due to isolation and incubation techniques. Determining the effective change ratios for cytokines released from stimulated and unstimulated PBMCs can account for cytokines released by unstimulated PBMCs, as these ratios are very dependent on the concentration of cytokines released from unstimulated PBMCs in the denominator. If altered PBMC function in FM participants is observed based on cytokine release from stimulated or unstimulated PBMCs, differences in PBMC function between FM participants and HCs should also still cause IL-6 and IL-10 concentrations to differ in unstimulated samples as the PBMCs from FM participants should still exhibit an altered ability to release cytokines. Many of the unstimulated samples from both FM participants and HCs in the present study also fell below the lower limit of detection for each of the IL-6 and IL-10 standards and had to be estimated based on the lowest level of detection. Data that fall around or below the lower limit of detection could be unreliable as there could be lots of variability around the detection limit. Future studies should consider using IL-6 and IL-10 standards with higher sensitivity and lower detection ranges to ensure unstimulated sample cytokine concentrations can be determined. In the present study, the sensitivities of the IL-6 and IL-10 assays were 4 pg/mL and 2 pg/mL, respectively, and the standard ranges of the IL-6 and IL-10 assays were 7.8-500 pg/mL and 3.9-250 pg/mL, respectively. For the analysis of IL-6 and IL-10 cytokines released from stimulated and unstimulated PBMCs, the relatively large standard error of the mean (SEM) could be due to many of the samples falling close to the lower limit of detection for the IL-6 and IL-10 assays, the small sample sizes used, and the large variation associated with individuals which is difficult to control. The large variability could partly be the reason significant differences were not observed between FM participants and HCs. The use of ELISAs to quantify released IL-6 and
IL-10 cytokine concentrations from stimulated and unstimulated PBMCs was also used as it is a better quantification method compared to the multiplex immunoassay.\textsuperscript{78,79} The higher released IL-6 and IL-10 concentrations in FM participants compared to HCs observed in the present study and its difference from previous studies observing lower released IL-6 and IL-10 concentrations in FM participants compared to HCs could also be investigated further as ELISA and multiplex immunoassay methods were respectively used, which could have influenced the results obtained.\textsuperscript{3,4}

In addition to differences in IL-6 and IL-10 concentrations released from stimulated PBMCs in individuals with FM compared to HCs, other cytokines could potentially also have altered concentrations released from stimulated PBMCs. In previous studies by Behm et al. 2012 and Wallace et al. 2015, the IL-8 cytokine had a blunted release response from stimulated PBMCs.\textsuperscript{3,4} IL-8 plays an important role in pain regulation and cognitive functions, which may also be an important indicator for the success of various FM treatment.\textsuperscript{3} Due to the diurnal effects of cytokine production for certain cytokines, the timing of blood draws for FM participants may also have affected the results obtained as different concentrations of cytokines could have been present at specific times.\textsuperscript{71} Differences in released cytokine concentrations could have also been seen after the final RSWT treatment depending on when the final blood draw was done following the treatment.

5.4 Influence of FM Subgroups and Comorbidities

Due to the lack of definitive diagnostic criteria for FM, individuals are usually diagnosed with FM by a rheumatologist after other conditions with similar symptoms have been eliminated. The development of the ACR Preliminary Diagnostic Criteria for FM screening tool has been widely accepted in clinical practice and it is the FM criteria utilized most often in the literature, but it is not always effective at accurately identifying FM.\textsuperscript{2} There have been Canadian guidelines released and some revisions to the 2010 ACR Preliminary Diagnostic Criteria in 2016 with intentions of improving FM classification and diagnosis.\textsuperscript{108} Although the 2010 ACR Preliminary Diagnostic Criteria screening tool takes into account the cognitive symptoms of individuals in addition to their physical pain symptoms, it is unable to accurately classify individuals into FM subgroups. The concept of FM subgroups is important as it can be used to develop and tailor
various treatment methods based on the symptoms experienced by individuals in a specific subgroup.\textsuperscript{26,27}

In the present study, the ACR criteria were used to identify individuals with FM to enroll them into the study; however, the FM participants were treated as a homogeneous group with no further classification into subgroups based on pain or symptom severity. Although FM subgroups have been recognized in the literature, there are no widely accepted proposed guidelines for FM subgroup classification.\textsuperscript{26,27} Some potential parameters used for the identification of FM subgroups could include identifying the severity of cognitive and physical symptoms such as depression and pain, respectively, as the presence of certain symptoms could greatly affect the treatment method of an individual with FM.\textsuperscript{26,28} The small sample size of 13 FM participants could have also made it difficult to assign individuals to FM subgroups as it might not be clear if differences in certain pain or cognitive symptoms could be normal variations within that subgroup, or if it could be a different subgroup all together. Although there were no significant differences for IL-6 or IL-10 release from PBMCs pre- and post-intervention for all FM participants, future work collecting pain and cognitive data from participants and combining it with observed trends in cytokine release could potentially be used to assign FM participants to subgroups. These groups could also be used to determine if RSWT is more effective at improving FM associated pain and symptoms in select subgroups that exhibit particular symptoms with certain severities.

The association of comorbidities with FM could also influence released cytokines detected from stimulated PBMCs. It is known that some comorbid conditions with FM have aberrant cytokine responses following PBMC stimulation and potential PBMC dysfunctions, including depression, chronic pain and chronic fatigue syndrome (CFS).\textsuperscript{10,56} Other conditions and factors for HCs and individuals with FM that could influence released cytokine concentrations from PBMCs are diabetes, smoking, and body mass index. The presence or absence of comorbid conditions in FM participants in the present study could potentially influence the stimulated released IL-6 and IL-10 from PBMCs. The present study did not take into account FM comorbidities that could be present in some FM participants, which could have had an effect on the results obtained from the quantification of released IL-6 and IL-10 cytokines from stimulated PBMCs. Research analyzing differences in cytokines released from stimulated
PBMCs in various FM comorbid conditions would be beneficial for future FM cytokine detection studies as it could help determine if there is a potential link to comorbidities in FM.

5.5 Factors that Affect PBMC Function and Cytokine Release

In the majority of studies analyzing differences in cytokine concentrations between FM individuals and HCs, only one sex was used. Many of these studies, however, also analyzed basal circulating cytokine levels instead of cytokines released from mitogen-stimulated PBMCs. One study analyzing cytokine concentrations released from stimulated PBMCs included both male and female FM individuals in their analyses, but there were significantly more females (89%) than males (11%). This uneven ratio of males and females could have potentially masked differences in released cytokine concentrations between sexes.

In Figure 2a, HC IL-6 concentrations from stimulated PBMCs were significantly different between sexes with males having a higher concentration than females. There was no significant difference observed for HC IL-6 concentrations from unstimulated PBMCs between sexes, as shown in Figure 2b. There was a significant difference in the IL-6 effective change ratio between males and females, however, with males having a higher effective change compared to males, as shown in Figure 4. Significant differences between sexes were also seen in Figures 3a and 3b for HC IL-10 concentrations from stimulated and unstimulated PBMCs, with males having higher concentrations than females for both sample analyses. For determining the released IL-10 concentrations for HC females, only six HC female samples were used compared to seven HC female samples used for determining the released IL-6 concentrations as there was not enough sample from one HC female participant for both assays. There was no significant difference in the IL-10 effective change ratio between males and females, as shown in Figure 5. Differences in HC IL-6 and IL-10 effective change ratios could be due to a potential correlation between sex hormones and PBMC function, where certain hormones may cause higher or lower concentrations of particular cytokines to be released. A study analyzing the relationship between certain sex hormone levels and secreted cytokines from stimulated PBMCs found some secreted cytokines were related to certain hormones, which differed between sexes. It is also known that there are interactions between the immune and endocrine systems, and the differences in cytokine concentrations between sexes could be a useful way to analyze this relationship. Taking into account hormonal status of participants could have affected the results.
obtained as the immune response can be influenced by hormones. Although there were only six to seven females and three males in the analysis in Figures 2, 3, 4, and 5, the differences in IL-6 and IL-10 concentrations from stimulated and unstimulated PBMCs and effective change ratios warrant further investigation with larger sample sizes. The effective change ratios of IL-6 and IL-10 cytokines from stimulated and unstimulated PBMCs is also greatly dependent on the unstimulated concentration denominator as it could determine if higher cytokine concentrations released from stimulated PBMCs is significant, or if the difference in cytokine concentrations between stimulated and unstimulated PBMCs is not very large. Although there was a lack of male participants with FM recruited in the present study, the participation of male individuals with FM could have also provided more insight regarding differences in IL-6 and IL-10 concentrations released from PBMCs to determine if differences were seen between sexes in FM individuals as well. Taking into account the height and mass of participants could have further supported differences seen in released cytokine concentrations between HC males and females, FM treatment and placebo groups, and FM treatment and HC groups.

It is also observed that the mean ages between HC males and females have a difference of 21 years, and HC males had a mean age of 48 years, which may introduce potential thoughts that age related differences are being observed instead of sex differences for IL-6 and IL-10 concentrations released from stimulated PBMCs. Although the analyses of IL-6 and IL-10 cytokines released from stimulated PBMCs between HC males and females are reflective of an assessment for immune system function, age difference is still a factor as immune function changes with age. With the knowledge of age-related and hormonal differences between males and females potentially affecting cytokines released from stimulated PBMCs, this provides insight for sex related differences of cytokine concentrations observed in the present study.\textsuperscript{63,66} It is also important to mention that although there were no differences in the results between HC females and all FM participants, the difference in mean ages between these two groups is 25.5 years, which is relatively large. HC females had a mean age of 27 years, while FM females had a mean age of 52.5 years. This large age difference between FM and HC females is important to note as it may potentially affect hormonal status, and in turn immune function, which may impact potential future studies. Future studies should be aimed at identifying potential relationships between stimulated PBMC function, age and sex.
5.6 Conclusion

The large number of individuals who suffer from FM and the many others who do not have an official diagnosis from a rheumatologist is cause for concern. The lack of a definitive diagnostic method for FM is even more alarming as FM symptom identification is not always effective. The results from the present study suggest there are no significant differences in IL-6 and IL-10 concentrations released from PBMCs in HCs and FM participants. These findings are in contrast to some previous studies where lower IL-6 and IL-10 concentrations released from stimulated PBMCs were observed in individuals with FM compared to HCs.\textsuperscript{3,4} Additionally, other studies that used ELISAs to quantify cytokine concentrations from stimulated PBMCs used the older 1990 ACR Preliminary Diagnostic Criteria for FM to identify individuals with FM, and studies that used the newer 2010 ACR Preliminary Diagnostic Criteria for FM to identify individuals with FM used multiplex immunoassays to quantify cytokine concentrations from stimulated PBMCs.\textsuperscript{3,4,7,3,74,77} The sample size of the present study was also not large enough, comorbidity information from FM participants was not collected, and other data measurements were not analyzed in the present study, including scores from the visual analog scale, the Baseline Pressure Tolerance Meter, the Quantitative Sensory Testing for hyperalgesia and allodynia, the Beighton Scoring Screen for hypermobility, and the Pain Catastrophizing Scale. There was insufficient evidence to establish if RSWT would benefit stimulated PBMC function in the present study, or if different FM subgroups would respond better to this treatment. Asai et al. 2001 demonstrated differences in cytokine release between males and females from PBMCs stimulated with lipopolysaccharide (LPS), and the present study found a significant difference in released IL-6 concentrations from PBMCs stimulated with PHA-P and IL-6 effective change between male and female HCs; however, various methods of stimulation may have different outcomes, as shown in Table 2.\textsuperscript{109} Due to the small FM participant and HC sample sizes, results from the present study were not significant and are classified as preliminary findings in a pilot study. Future work would benefit from using larger FM and HC sample sizes and stricter screening and inclusion criteria to potentially identify FM subgroups, and to evaluate the influence of FM comorbidities.
5.7 Future Directions

The present study provides many opportunities for future research to analyze differences in cytokine concentrations from stimulated PBMC in individuals with FM as a diagnostic method, and also for evaluating the effectiveness of RSWT as a FM treatment option. Future research could create customized approaches for treating individuals with FM to reduce specific FM symptoms associated with FM subgroups, and to identify potential inflammatory FM subgroups. For analyzing cytokine concentrations released from stimulated PBMCs, isolating PBMC subpopulations could determine if cytokine production and release in certain cell types, such as those from T-cells, are altered. Some PBMCs release specific or more types of cytokines than others after stimulation with a certain mitogen, and analyzing their release could identify potential cellular dysfunctions. Flow cytometry and intracellular staining could then be used to analyze various characteristics of dysfunctional PBMC subpopulations to determine if there are differences compared to HCs. The identification of specific PBMC subpopulation functions could also aid in developing treatment methods that target these certain cell types. While the current study did not observe any significant differences regarding improved PBMC function in FM patients from RSWT treatment, future studies can use larger sample sizes to identify FM subgroups and determine which subgroups responded better to RSWT treatments.

In addition to studies analyzing cytokines as biomarkers to diagnose FM, future studies can evaluate different analysis techniques as FM diagnostic approaches. There has recently been a study published that identified individuals with FM with high accuracy using vibrational spectroscopy and metabolites unique to these individuals. Other studies have used gene expression analysis to determine genes that are correlated to individuals with FM. These studies show promising future FM diagnostic research using different analysis approaches.
Chapter 6. Bibliography


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Chapter 7. Appendix

7.1 List of Abbreviations

ACR – American College of Rheumatology
ANOVAs – Analysis of Variances
CFR – Code of Federal Regulations
CFS – Chronic Fatigue Syndrome
CNS – Central Nervous System
Con A – Concanavalin A
CRH – Corticotrophin-Releasing Hormone
CWP – Chronic Widespread Pain
COX-1 – Cyclooxygenase-1
COX-2 – Cyclooxygenase-2
CO₂ – Carbon Dioxide
Dex – Dexamethasone
DHEA-S – Dehydroepiandrosterone Sulfate
ELISA – Enzyme-Linked Immunosorbent Assay
ESWT – Extracorporeal Shockwave Therapy
FBCT – Functional Biomarker Challenge Test
FCIII – Fetal Clone III
FM – Fibromyalgia
FDA – Food and Drug Administration
X g – G-force
HC – Healthy Control
HDRS – Hamilton Depression Rating Scale
IBS – Irritable Bowel Syndrome
IFN-γ – Interferon Gamma
IL-1β – Interleukin-1 Beta
IL-1RA – Interleukin 1 Receptor Antagonist
IL-1 – Interleukin-1
IL-2 – Interleukin-2
IL-2r – Interleukin-2 receptor
IL-3 LA – Interleukin-3-Like Activity
IL-4 – Interleukin-4
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-10 – Interleukin-10
IL-13 – Interleukin-13
LPS – Lipopolysaccharide
LSD – Least Significant Difference
LU – Lakehead University
MC – Mast Cell
MCAS – Mast Cell Activation Syndrome
MCP – Monocyte Chemoattractant Protein
MDC – Macrophage Derived Chemokine
MDD – Major Depressive Disorder
MIP-1α – Macrophage Inflammatory Protein-1 Alpha
MIP-1β – Macrophage Inflammatory Protein-1 Beta
NaPyr – Sodium Pyruvate
NK – Natural Killer
NOSM – Northern Ontario School of Medicine
NSAID – Non-Steroidal Anti-Inflammatory Drug
OD – Optical Density
PBMC – Peripheral Blood Mononuclear Cell
PBS – Phosphate Buffered Saline
PHA – Phytohaemagglutinin
PHA-E – Phytohaemagglutinin Erythrocyte
PHA-L – Phytohaemagglutinin Leukocyte
PHA-P – Phytohemagglutinin-P
PMA – Phorbol Myristate Acetate
POTS – Postural Orthostatic Tachycardia Syndrome
PTSD – Post Traumatic Stress Disorder
REB – Research Ethics Board
RA – Rheumatoid Arthritis
RPMI – Roswell Park Memorial Institute
RSWT – Radial Shockwave Therapy
SEM – Standard Error of the Mean
sIL-2R – Serum IL-2 Receptor
SJCG – St. Joseph’s Care Group
SP – Substance P
SLE – Systemic Lupus Erythematosus
SSRI – Selective Serotonin Reuptake Inhibitor
TBRHSC – Thunder Bay Regional Health Sciences Centre
TCR – T-cell Receptor
TGF-β1 – Transforming Growth Factor Beta 1
TNF-α – Tumor Necrosis Factor Alpha
7.2 2010 ACR Preliminary Diagnostic Criteria for FM

AMERICAN COLLEGE OF RHEUMATOLOGY (ACR)
PRELIMINARY DIAGNOSTIC CRITERIA FOR FIBROMYALGIA

The information contained on this form was derived from Wolfe F, Clauw DJ, Fitzcharles MA, et al. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. Arthritis Care Res. 2010;62(5):600-610.

PART 1: WIDESPREAD PAIN INDEX

HOW TO CALCULATE THE PATIENT’S WIDESPREAD PAIN INDEX (WPI)

1. Using the list of 19 body areas, identify the areas where the patient felt pain over the past week. As a visual aid, front/back body diagrams are included.
   — Each area identified on the list counts as 1

2. Total the number of body areas (the WPI score can range from 0 to 19).

Write the patient’s WPI score here: ____________ .

Identify the areas where the patient felt pain over the past week:

- Shoulder girdle, left
- Shoulder girdle, right
- Upper arm, left
- Upper arm, right
- Lower arm, left
- Lower arm, right
- Lower leg, left
- Lower leg, right
- Hip (buttock), left
- Hip (buttock), right
- Upper leg, left
- Upper leg, right
- Jaw, left
- Jaw, right
- Neck
- Upper back
- Lower back
- Abdomen
- Chest
- Hip (buttock)
- Upper arm
- Lower arm
- Lower back
- Lower leg
- Upper leg
- Neck
- Shoulder girdle
- Jaw
- Lower leg
- Upper arm
- Upper back
- Lower back
- Abdomen
- Chest

FRONT SIDE

BACK SIDE
PART 2A: SYMPTOM SEVERITY SCALE (LEVELS OF SEVERITY)

HOW TO MEASURE THE PATIENT’S LEVEL OF SYMPTOM SEVERITY

1. Using a scale of 0 to 3, indicate the patient’s level of symptom severity over the past week in each of the 3 symptom categories. Choose only 1 level of severity for each category.
   — The score is the sum of the numbers that correspond to the severity levels identified in all 3 categories

2. Total the scale numbers for all the 3 categories and write the number here: ____________________

<table>
<thead>
<tr>
<th>Fatigue</th>
<th>Waking unrefreshed</th>
<th>Cognitive symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ 0 = No problem</td>
<td>□ 0 = No problem</td>
<td>□ 0 = No problem</td>
</tr>
<tr>
<td>□ 1 = Slight or mild problems; generally mild or intermittent</td>
<td>□ 1 = Slight or mild problems; generally mild or intermittent</td>
<td>□ 1 = Slight or mild problems; generally mild or intermittent</td>
</tr>
<tr>
<td>□ 2 = Moderate; considerable problems; often present and/or at a moderate level</td>
<td>□ 2 = Moderate; considerable problems; often present and/or at a moderate level</td>
<td>□ 2 = Moderate; considerable problems; often present and/or at a moderate level</td>
</tr>
<tr>
<td>□ 3 = Severe; pervasive, continuous, life-disturbing problems</td>
<td>□ 3 = Severe; pervasive, continuous, life-disturbing problems</td>
<td>□ 3 = Severe; pervasive, continuous, life-disturbing problems</td>
</tr>
</tbody>
</table>

PART 2B: SYMPTOM SEVERITY SCALE (OTHER SOMATIC SYMPTOMS)

HOW TO DETERMINE THE EXTENT OF THE PATIENT’S OTHER SOMATIC SYMPTOMS

Using the symptoms list on the following page, determine the extent of other somatic symptoms the patient may have experienced over the past week.

1. Determine the quantity of somatic symptoms using the list on the following page.

2. Using your best judgment, calculate the score that matches the quantity of those somatic symptoms and write the number here: ____________________

Add the scores from Parts 2a and 2b (the Symptom Severity score, or SS score, can range from 0 to 12).
Write the patient’s SS score here: ____________________
### OTHER SYMPTOMS

<table>
<thead>
<tr>
<th>Muscle pain</th>
<th>Depression</th>
<th>Itching</th>
<th>Dry eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irritable bowel syndrome</td>
<td>Constipation</td>
<td>Wheezing</td>
<td>Shortness of breath</td>
</tr>
<tr>
<td>Fatigue/tiredness</td>
<td>Pain in upper abdomen</td>
<td>Raynaud’s</td>
<td>Loss of appetite</td>
</tr>
<tr>
<td>Thinking or memory problem</td>
<td>Nausea</td>
<td>Hives/welts</td>
<td>Rash</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td>Nervousness</td>
<td>Ringing in ears</td>
<td>Sun sensitivity</td>
</tr>
<tr>
<td>Headache</td>
<td>Chest pain</td>
<td>Vomiting</td>
<td>Hearing difficulties</td>
</tr>
<tr>
<td>Pain/cramps in abdomen</td>
<td>Blurred vision</td>
<td>Heartburn</td>
<td>Easy bruising</td>
</tr>
<tr>
<td>Numbness/tingling</td>
<td>Fever</td>
<td>Oral ulcers</td>
<td>Hair loss</td>
</tr>
<tr>
<td>Dizziness</td>
<td>Diarrhea</td>
<td>Loss/change in taste</td>
<td>Frequent urination</td>
</tr>
<tr>
<td>Insomnia</td>
<td>Dry mouth</td>
<td>Seizures</td>
<td></td>
</tr>
</tbody>
</table>

Based on the quantity of symptoms, the patient’s score is:

- ☐ 0 = No symptoms
- ☐ 1 = Few symptoms
- ☐ 2 = A moderate number of symptoms
- ☐ 3 = A great deal of symptoms

### WHAT THE PATIENT’S SCORE MEANS

The patient’s WPI score (Part 1): ____________

The patient’s SS score (Parts 2a and 2b): ____________

A PATIENT MEETS THE DIAGNOSTIC CRITERIA FOR FIBROMYALGIA IF THE FOLLOWING 3 CONDITIONS ARE MET:

1a. The WPI score (Part 1) is greater than or equal to 7 and the SS score (Parts 2a and 2b) is greater than or equal to 5.

OR

1b. The WPI score (Part 1) is from 3 to 6 and the SS score (Parts 2a and 2b) is greater than or equal to 9.

2. Symptoms have been present at a similar level for at least 3 months.

3. The patient does not have a disorder that would otherwise explain the pain.

7.3 Flow of FM Participants through Each Stage of the Present Study

Figure 17. Flow of fibromyalgia (FM) participants through each stage of the present study.
7.4 Blood Sample Processing and Storage

7.4.1 Blood Sample Processing and Storage – Day 1

Materials:
- BD Vacutainer CPT Tube
- 2X vacutainer adapters for the table top centrifuge
- Table top centrifuge
- Vacutainer water balance
- Pasteur Pipette
- Pipette bulb
- 2X 15 mL conical centrifuge tubes with caps
- Conical centrifuge tube water balance
- 2X conical centrifuge tube adapters for the table top centrifuge
- Phosphate buffered saline (PBS) solution
- 25 mL sterile serological pipette
- 10 mL sterile serological pipette
- Pipetting aid
- RPMI+FCIII media
- Bead heater set at 37˚C
- Tube rack
- 200-1000 µL pipette with appropriate tips
- 1.5 mL microcentrifuge tube
- Hemocytometer slide with appropriate cover slip
- Nikon H550S phase contrast microscope
- 2-20 µL pipette with appropriate tips
- 20 µL to 200 µL pipette with appropriate tips
- PHA-P
- Waste container
- Biohazard disposal bag
- Kim Wipes
- 70% ethanol
- Paper towel
- Ethanol resistant marker
- 24 well plate (or 12 well plate depending on media volume needed to obtain 1 million cells per mL)
- Incubator at 37°C with 5% CO₂

Methods:
1. After a vial of the participant’s blood has been collected, store the tube upright at room temperature. Let the tube sit for about one hour before it is centrifuged. The tube must be centrifuged within two hours post draw.
2. After about an hour, invert the tube 8-10 times to remix the blood sample.
3. Obtain the appropriate vacutainer adapters for the table top centrifuge. Place the blood sample and a water balance into these adapters, ensure they are balanced, and centrifuge for 25 minutes at 1500Xg (3000 rpm).
4. Following centrifugation, transfer the vacutainer of blood into the biosafety cabinet (BSC). Use a Pasteur pipette and bulb to remove the clear/whiteish layer of cells below the plasma and above the density solution in the tube. Transfer this solution into a 15 mL centrifuge tube and add PBS to a final volume of 15 mL using a 25 mL sterile pipette. Invert the tube 5 times to mix.
5. Obtain the appropriate conical centrifuge tube adapters for the table top centrifuge. Place the 15 mL centrifuge tube and water balance into these adapters, ensure they are balanced, and centrifuge for 15 minutes at 300Xg (1400 rpm).
6. During this time, pipette the appropriate amount of media (RPMI+FCIII etc) into a 15 mL centrifuge tube and warm in the bead heater. The amount of media added to the tube is based on the number of vials of blood being processed. There will be 7 mL of media used for each vial of blood (2 wells per vial with 3 mL in each well and 1 mL extra).
7. Obtain the PHA-P in the 1.5 mL microcentrifuge tube from the -20°C freezer and place into the bead heater to thaw. Transfer to the BSC once thawed.
8. After centrifugation, place the centrifuge tube into the BSC. Pour off the supernatant and resuspend the pellet by gently tapping the tube.
9. Add PBS to a final volume of 10 mL in the tube using a 10 mL pipette, and invert 5 times to mix.

10. Centrifuge the tube in the table top centrifuge using the appropriate adapters and water balance for 10 minutes at 300Xg (1400 rpm).

11. Obtain the media in the 15 mL centrifuge tube from the bead heater and place in the BSC.

12. Following centrifugation, place the tube back into the BSC and pour off the supernatant. Use a 1000 µL pipette to resuspend the pellet in 1 mL of media to count.

13. In a 1.5 mL microcentrifuge tube, dilute the resuspension to 1:10 to count. Do this by pipetting 20 µL of the cell suspension solution into the tube, and then pipetting 180 µL of PBS into the same tube. This gives a final volume of 200 µL.

14. Count the cells in the hemocytometer by transferring 10 µL of the 1:10 dilution to each side of the slide. Count the cells found in all 9 squares on both sides of the slide.

15. Add the cell counts from both sides and average them, and divide this average by 9 (the amount of squares counted on one side). Multiply this number by $10^4$ (a constant), and multiply this number by the dilution factor (10 in this case since the dilution is 1:10). The resulting number will be the total amount of cells per mL in the sample.

16. To obtain a concentration of 1 million cells per mL, multiply the volume left in the 15 mL centrifuge tube (980 µL in this case since the resuspension volume was 1 mL and 20 µL was taken out to count) by the amount of cells per mL in the sample. Divide this number by $1 \times 10^6$ cells/mL as we want to obtain 1 million cells per mL. This is the total amount of media that needs to be added to the cells to get 1 million cells per mL. Since there is already 980 µL of volume in the tube, subtract this volume from the total amount of media needed to obtain 1 million cells per mL.

17. Use a 200-1000 µL and a 2-20 µL pipette to pipette the appropriate amount of media into the 1 mL suspension to obtain 1 million cells per mL.

18. Divide the total amount of media that needs to be added to the cells to get 1 million cells per mL by 2 since we need a stimulated and an unstimulated well.

19. Once the volume of media with cells that needs to go into each well is obtained, pipette this volume into two wells labeled stimulated and unstimulated on a 24 well plate using the appropriate pipettes and tips. (Note: if the volume in each well exceeds about 1.5 mL, divided the total volume by 4, ie: if the total volume is greater than 3 mL, divide it by 4
so each well has 0.75 mL. This will result in 2 stim and 2 unstim wells instead of the normal 1 stim and 1 unstim wells per sample. A 12 well plate was used instead once to allow a larger surface area for the media to come into contact with the air.)

20. After the cells have been seeded into the wells, treat the stimulated labeled well with PHA-P. To get a final PHA-P concentration of 10 µg/mL from the stock solution of 1 mg/mL, multiply the volume in each well by 10 µg/mL, divide this value by 1000 µg/mL, and convert from mL to µL. Pipette the appropriate amount of PHA-P into the stimulated labeled well.

21. Incubate the cells at 37°C at 5% CO₂ for 18 hours.

7.4.2 Blood Sample Processing and Storage – Day 2

Materials:
- 24 well plate (or 12 well plate) containing stimulated and unstimulated cells
- Nikon H550S phase contrast microscope
- +4X 1.5 mL microcentrifuge tubes
- 200-1000 µL pipette with appropriate tips
- Temperature controlled table top centrifuge for 1.5 mL microcentrifuge tubes
- Biohazard disposal bag
- Kim Wipes
- 70% ethanol
- Ethanol resistant marker
- Incubator at 37°C with 5% CO₂

Methods:
1. Turn on the centrifuge and let cool to 4°C.
2. After the 18 hours, check out the wells in the 24 well plate under the microscope and observe how the stimulated cells look compared to the unstimulated cells.
3. Take the entire media volume from each well and pipette into two 1.5 mL microcentrifuge tubes. Centrifuge for 10 minutes at 16000Xg at 4°C. Make sure the centrifuge lid is on tight, set “fast temp” and stick the hinge on the tube facing out.
4. Following centrifugation, pipette the supernatant from each tube into new 1.5 mL tubes and invert to mix.

5. Make an appropriate amount of 220 µL aliquots and 30 µL aliquots for each well (stimulated and unstimulated) in 1.5 mL microcentrifuge tubes. Make as many 220 µL aliquots as possible first, and then make 30 µL aliquots.

6. Pipette any remaining supernatant volume into another 1.5 mL microcentrifuge tube and note the volume.

7. Label the tubes with the sample name and date, and put all of them into the appropriate rack and box in the -80°C freezer.
7.5 Blood Sample Preparation for ELISA

Materials:
- Ice bucket with ice
- Human IL-6 or IL-10 ELISA MAX Capture Antibody vial (300 µL)
- 5X Coating Buffer A (5X CBA)
- 2 mL or 15 mL centrifuge tube
- Ethanol resistant marker
- NUNC Maxisorp 96 Microwell Plate
- Plate sealer
- Brown PCR squeegee
- 200-1000 µL pipette with appropriate tips
- 2-20 µL pipette with appropriate tips
- 50-200 µL pipette with appropriate tips
- 50 mL conical centrifuge tube, or 50 mL beaker
- Tube rack
- Table top centrifuge
- 2 mL microcentrifuge tube water balance
- Double distilled water (ddH₂O)
- Scalpel
- Aluminum foil

Methods:
1. Place the capture antibody, 5X Coating Buffer A (CBA), and labeled 2 mL or 15 mL centrifuge tube into a bucket of ice.
2. Mix the capture antibody by gently inverting. NEVER VORTEX AN ANTIBODY. To get the solution off the vial lid, centrifuge the vial with a balance for 2 seconds.
3. Determine how much well coating solution is needed to coat the desired amount of plate wells. Do this by finding the number of wells needed for each sample and their dilutions (ran in duplicates), adding an extra well to account for error, and multiplying by 100 µL as this is the amount needed in each well.
4. Since the capture antibody has to be diluted 1:200, divide the volume of coating solution needed by 200 to get the amount of capture antibody needed in µL.

5. Since the capture antibody is diluted with 1X CBA, subtract the amount of capture antibody needed from the volume of coating solution needed. This is the amount of 5X CBA and ddH₂O needed. Divide this value by 5 to get the amount of 5X CBA needed. Multiply the amount of 5X CBA needed by 4 to get the amount of ddH₂O needed.

6. Fill a 50 mL conical centrifuge tube or beaker with ddH₂O and label.

7. Pipette the appropriate amounts of 5X CBA and ddH₂O into a 2 mL or 15 mL centrifuge tube.

8. Add the appropriate amount of the capture antibody to the centrifuge tube. Use a 1000 µL pipette to gently mix the solution.

9. Obtain the ELISA plate (new or partially used), being careful not to touch the bottom. Squeegee sealing film onto the plate to ensure a complete seal is formed.

10. Use a scalpel to cut away a section of sealing film for the wells to be used. Save this section as it will be used to re-seal the rows.

11. Pipette 100 µL of the coating solution into each well.

12. Place the section of sealing film back over the rows filled with solution, and use the squeegee to form a tight seal to ensure the solution will not evaporate.

13. Label the plate only in the area where the sealing film was removed as the rest of the plate can be used in future experiments. Put the date, initials, and “IL-6 or IL-10 ELISA”.

14. Place the plate onto aluminum foil to ensure the plate bottom does not touch the fridge, and incubate at 4°C for 16-18 hours.
7.6 Blood Sample Analysis using ELISA

Materials:
- Ice bucket with ice
- ELISA plate
- Aluminum foil
- Human IL-6 Standard (23 ng) or IL-10 Standard (27 ng)
- 1000X Avidin-Horseradish Peroxidase (HRP) (60 µL)
- Human IL-6 or IL-10 ELISA MAX Detection Antibody vial (300 µL)
- 5X Assay Diluent A (5X ADA)
- Substrate Solution A (SSA)
- Substrate Solution B (SSB)
- Stop Solution (sulfuric acid)
- Phosphate-buffered saline (PBS) powder
- 1L graduated cylinder
- Weigh boat
- Scoopula
- Weigh scale
- Distilled water (dH₂O)
- Stir bars
- Stir plate
- 1 L screw top bottle
- Labeling tape
- Ethanol resistant marker
- 500 mL beaker
- 200 mL or 500 mL graduated cylinder
- Tween-20
- Syringe
- Pipette basin
- Multichannel pipettor
- 300 mL pipette tips
- 15 mL or 50 mL centrifuge tube
- 1.5 mL microcentrifuge tubes
- Tube rack
- Brown PCR squeegee
- Plate shaker
- 200-1000 µL pipette with appropriate tips
- 2-20 µL pipette with appropriate tips
- 50-200 µL pipette with appropriate tips
- 96 Microwell Plate Balance
- 2 mL and/or 15 mL centrifuge tubes
- Table top centrifuge
- 2 mL microcentrifuge tube water balance
- 5 mL seriological pipette
- 10 mL or 25 mL seriological pipette
- Pipetting aid

Methods:
1. Bring the 5X Assay Diluent A (ADA) and Human IL-6 or IL-10 standard to room temperature, which should take about 30 minutes.
2. Fill a 1 L graduated cylinder with dH₂O.
3. Make the 1X phosphate buffered saline (PBS) solution by adding 9.88 g of PBS powder, 1 L of dH₂O and a large stir bar to a 1 L screw top container, and placing onto a stir plate until the PBS completely dissolves into solution. Store at room temperature.
4. Make the wash buffer by measuring the amount of PBS needed plus ~50 mL extra into a 200 mL or 500 mL graduated cylinder. Pour the PBS into a 500 mL beaker with a stir bar, and add Tween-20 using a syringe so it is at a concentration of 0.05%. For 100 mL of PBS, add 0.05 mL of Tween-20. Overfill the syringe and dispense the amount needed, ensuring there are no air bubbles. Place onto a stir plate being careful not to make foamy bubbles.
5. Determine the amount of 1X ADA needed for Parts I to V, and make this solution in a 15 mL or 50 mL centrifuge tube. Since the 5X ADA has to be diluted to 1X ADA, take
the amount of 1X ADA needed and divide by 5. This is the amount of 5X ADA needed. Multiply the amount of 5X ADA needed by 4 to determine the amount of PBS needed.

6. **Part I.** Determine the amount of 1X ADA needed to block non-specific binding. Do this by finding the number of wells needed for each sample and their dilutions (ran in duplicates), adding an extra well to account for error, and multiplying by 200 µL as this is the amount needed in each well.

7. Obtain the ELISA plate from the fridge after the 16-18 hour incubation.

8. Obtain the wash buffer and pour some into the pipette basin. Wash the appropriate wells by removing the section of sealing film, pouring out the solution in the wells into the sink, banging the plate onto paper towel, and pipetting 300 µL of wash solution into the wells using a multichannel pipettor. Repeat this procedure to wash out the wells 4 times.

9. Pipette 200 µL of the 1X ADA into each well, use the squeegee to re-seal the plate, and place onto a plate shaker at 500 rpm with a 0.3 circular orbit for 1 hour. Place another empty plate on the other side to act as a balance.

10. **Part II.** Determine the amount of 1X ADA needed for the standards based on the number of wells used as follows:

    a. Add 200 µL of 1X ADA to the lyophilized IL-6 or IL-10 standard in the vial and allow it to reconstitute at room temperature for 15-20 minutes. Pipette the solution into a 1.5 mL tube and vortex briefly to mix. Make nine or ten 20 µL aliquots of the standard in 1.5 mL tubes, and store in the “IL-6 or IL-10 ELISA Samples Fibro” box in Rack 4 of the -80°C freezer. *Once the standard is reconstituted and aliquoted, this step is not needed.*

    b. Dilute 4.3 µL of the IL-6 standard stock solution with 995.7 µL of 1X ADA to make the first standard. *For IL-10, perform an initial 1:10 dilution by adding 10 µL stock solution with 90 µL of 1X ADA. Add 18.5 µL of the 1:10 dilution to 981.5 µL of 1X ADA, which will be the top standard.*

    c. Pipette 500 µL of 1X ADA into each of the seven 1.5 mL tubes which will form the standard curve and blank. The total amount of 1X ADA used is 3.5 mL.

    d. Perform six serial dilutions by transferring 500 µL from each previous dilution into the next tube to make the seven standards.
11. **Part III.** Determine the amount of 1X ADA needed to dilute the samples based on the number of samples used and how much they need to be diluted. Prepare the dilutions for each of the samples in 1.5 mL tubes using 1X ADA.

12. After the hour incubation, wash the wells 4 times with wash buffer as outlined above.

13. Create a plate map of where each sample will go, and pipette 100 µL of each sample and standard into the appropriate wells. Re-seal the plate using the squeegee, and incubate as outlined above for 2 hours.

14. After the 2 hour incubation, wash the wells 4 times with wash buffer as outlined above.

15. **Part IV.** Determine the amount of 1X ADA needed to dilute the detection antibody based on the number of wells used as follows:

    a. Place the detection antibody and a labeled 2 mL or 15 mL centrifugation tube in ice.
    b. Mix the detection antibody by inverting. NEVER VORTEX AN ANTIBODY. To get the solution off the vial lid, centrifuge the vial with a balance for 2 seconds.
    c. Determine the amount of solution needed for the number of wells to be used. Do this by finding the number of wells needed for each sample and their dilutions (ran in duplicates), adding an extra well to account for error, and multiplying by 100 µL as this is the amount needed in each well.
    d. Since the detection antibody has to be diluted 1:200, divide the total solution volume needed by 200 to get the amount of detection antibody needed in µL.
    e. Since the detection antibody is diluted with 1X ADA, subtract the amount of detection antibody needed from the total solution volume. This is the amount of 1X ADA needed.
    f. Pipette the appropriate amount of 1X ADA into a 2 mL or 15 mL centrifugation tube and add the detection antibody. Use a 1000 µL pipette to gently mix the solution.

16. Pipette 100 µL of the diluted detection antibody solution into each well, seal with the squeegee, and incubate for an hour as outlined above.

17. After the hour incubation, wash the wells 4 times with wash buffer as outlined above.

18. **Part V.** Determine the amount of 1X ADA needed to dilute the Avidin-Horseradish peroxidise (HRP) based on the number of wells used as follows.

    a. Determine the amount of solution needed for the number of wells used. Do this by finding the number of wells needed for each sample and their dilutions (ran in
duplicates), adding an extra well to account for error, and multiplying by 100 µL as this is the amount needed in each well.

b. Mix the Avidin-HRP by gently inverting. DO NOT VORTEX. To get the solution off the vial lid, centrifuge the vial with a balance for 2 seconds.

c. Since the Avidin-HRP has to be diluted 1:1000, divide the total solution volume needed by 1000 to get the amount of Avidin-HRP needed in µL.

d. Since the Avidin-HRP is diluted with 1X ADA, subtract the amount of Avidin-HRP needed from the total solution volume. This is the amount of 1X ADA needed.

e. Pipette the appropriate amount of 1X ADA into a 2 mL or 15 mL centrifuge tube and add the appropriate amount of Avidin-HRP. Use a 1000 µL pipette to mix.

19. Pipette 100 µL of the diluted Avidin-HRP solution into each well, seal with the squeegee, and incubate for 30 minutes as outlined above.

20. After the hour incubation, wash the wells 5 times with wash buffer as outlined above. On the last wash, let the wash buffer soak in the wells for 1 minute to ensure the background is minimized when the plates are read.

21. Determine the amount of 3,3’,5,5’–tetramethylbenzidine (TMB) substrate solution needed based on the number of wells as follows:

   a. Find the number of wells needed for each sample and their dilutions (ran in duplicates), add an extra well to account for error when making up the solution, and multiply by 100 µL as this is the amount needed in each well.

   b. Divide the amount of TMB solution needed by 2 to get the amount of Substrate Solution A (SSA) and Substrate Solution B (SSB) needed in equal parts.

   c. Pipette the appropriate amounts of SSA and SSB into a 2 mL or 15 mL centrifuge tube. Use a 1000 µL pipette to gently mix the solution.

22. Pipette 100 µL of the TMB substrate solution into each well, DO NOT seal the plate, and incubate in the dark for 15 minutes without shaking for the IL-6 assay. For IL-10 assay, incubate in the dark for 30 minutes.

23. During the incubation period, set up the computer as follows:

   a. Turn the plate reader on and click the “Microplate Readers” user

   b. Click the “KC4” icon on the desktop
c. Click “Read” on the top toolbar. In the new window that comes up, select “End Point,” and “Wavelengths=2.”

d. Choose 450 nm and 570 nm as the wavelengths to read the sample

   i. If the desired wavelength is not in the drop-down menu, type it in and click “Calibrate.” No plate needs to be in the reader for this step.

e. Choose “Read Mode=Normal,” and “96 Well Plate.”

f. Select which wells you want to read (A1-H12 reads the whole plate starting at the top going from left to right).

24. Determine the amount of stop solution needed for the number of wells used. Do this by finding the number of wells needed for each sample and their dilutions (ran in duplicates), adding an extra well to account for error, and multiplying by 100 µL as this is the amount needed in each well.

25. Pipette the appropriate amount of stop solution into a 2 mL or 15 mL centrifuge tube, and add 100 µL to each well after the incubation period.

26. Remove the sealing film covering the unused wells, and place into the plate reader ensuring the plate corner with the A1 well matches up with the A1 marking on the plate reader (top left part of plate goes into top right side of plate reader).

27. Run the plate reader, copy the data obtained for readings at 450 nm, 570 nm and the Delta Optical Density (OD) into Excel, and save both the KC4 and Excel files.

28. Create a standard curve graph along with the accompanying equation for later analysis.

29. Reseal the unused wells with sealing film using the squeegee, pour out the solution in the wells, and rinse the wells with distilled water. Bang the plate onto paper towel to dry.
7.7 RPMI Media Preparation

Materials:
- HyClone Roswell Park Memorial Institute (RPMI) 1640 Medium with 2.05 mM L-GLutamine additive, 500 mL
- Fetal Clone III (FCIII) 50 mL aliquot in -20°C freezer
- Penicillin-Streptomycin (pen/strep) 5 mL aliquot in -20 °C freezer
- Sodium pyruvate (NaPyr)
- 50 mL centrifuge tube
- Tube rack
- Ethanol resistant marker
- Ethanol
- Kim Wipes
- Bead heater set at 37°C
- Biohazard disposal bag
- Pipetting aid
- 2 X 25 mL sterile serological pipette
- 5 mL sterile serological pipette

Methods:
1. Obtain the pen/strep 5 mL aliquot in the 10 mL centrifuge tube and the FCIII 50 mL aliquot from the -20°C freezer and place into a bead heater set at 37°C. Check on the tubes ever 15 minutes, gently inverting each time, until they are thawed.
2. Once the tubes are thawed, place into the fridge until ready to use.
3. Place a 50 mL centrifuge tube in the biosafety cabinet (BSC) and label as “RPMI 1640 media, date, initials” and expiry indicated on the original RPMI 1640 bottle.
4. Obtain the RPMI 1640 media, NaPyr, pen/strep, and FCIII from the fridge and place into the BSC.
5. Use a 25 mL pipette to remove 50 mL of media from the original bottle into the labeled 50 mL centrifuge tube.
6. Use a 5 mL pipette to add 5 mL of NaPyr to the original media bottle, and use the same pipette to add 5 mL of pen/strep to the media as well.
7. Use a 25 mL pipette to add 50 mL of FCIII to the media. Mix by pipette up and down 20 times.

8. Label the media as “10% FCIII, 1% pen/strep, 1% NaPyr, initials and date.”

9. Place the 50 mL of RPMI 1640 and the supplemented media into the Styrofoam box in the fridge, as the media is light sensitive.