Inflammatory modulators of central sensitization in fibromyalgia and temporomandibular disorders: a pilot study

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Abstract

Inflammatory modulators of central sensitization in fibromyalgia and temporomandibular disorders: a pilot study

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Background: This thesis is expanding on a previous pilot study that was entitled *G protein-coupled Receptor Kinase 2 (GRK2) and Toll-like Receptors as Regulators of Central Sensitization in Fibromyalgia and Chronic Temporomandibular Disorders,* by Drangsholt et. al. 2018 (designated here as "pilot study-A"). Fibromyalgia (FM) and chronic temporomandibular disorders (TMD) are both chronic pain syndromes that are challenging to manage, especially since they are not explained by tissue lesions. They both feature an increased activity of pain pathways in the central nervous system, i.e. central sensitization (CS). However, the molecular mechanisms underlying CS are unclear, which hampers the development of targeted treatments. Because of the strong evidence that implicates the immune system in modulating central pain pathways, pilot study-A examined the expression levels of GRK2, a negative modulator of the immune response, and the toll-like receptors' (TLR) downstream cytokine interleukin 1-beta (IL1β), a positive modulator of the immune response. Importantly, both inflammatory modulators are found centrally in glial cells and in peripheral blood mononuclear cells (PBMCs).

Our ability to measure these molecules in PBMCs is clinically important because we can't measure them in glial cells of living human subjects. In pilot study-A, the researcher successfully enrolled 11 TMD patients, 9 fibromyalgia (FM) patients and 18 controls to examine the levels of GRK2 and IL1β after TLR stimulation in blood samples from these three groups. The hypotheses of that study were: 1) PBMCs from FM and chronic TMD patients have lower basal GRK2 levels. 2) These levels are negatively correlated with indices of CS. 3) the blood of FM and TMD patients displays exaggerated IL1β production after TLRs stimulation. 4) IL1β levels correlate positively with indices of CS. In a preliminary analysis of the recruited patients, pilot study-A exhibited a negative correlation between Nociceptive Flexion Reflex Threshold (NFRT) and IL1β level following TLR stimulation, which was in line with one of the hypotheses of the study.

Aims: With this thesis I worked to accomplish two aims: 1) Complete the goal of pilot study-A of recruiting patients to reach 18 recruits for the TMD and FM groups and to analyze both the levels of IL1β after TLR stimulation and basal GRK2 levels in all groups at $n = 18$ and test the hypotheses of the previous study. 2) Make use of the full complement of blood samples at $n = 18$ for each of the three study groups to expand on pilot study-A by measuring other molecular candidates of the immune system that I hypothesize to modulate CS. Specifically, I wished to look at the protein levels of tumor necrosis factor- alpha (TNFα) which is downstream of TLR activation in both glial cells and PBMC.

Hypotheses: The pro-inflammatory cytokines (IL1β and TNFα) are expected to be elevated in FM and TMD samples relative to controls post-TLR stimulation, while the anti-inflammatory GRK2 is expected to be lower in FM and TMD samples relative to controls. With respect to

indices of CS, I expected to find a positive correlation with pro-inflammatory cytokine expression and a negative correlation with GRK2 levels.

Methods**:** I successfully added 2 subjects to the TMD research group (total $n = 13$) and 8 subjects to the FM research group (total $n = 17$) to the cohort from pilot study-A. I assessed CS and production of TNFα and IL1β post-TLR stimulation in vitro. I also isolated PBMCs to acquire protein lysates which underwent a Western Blot assay to quantify GRK2 levels.

Results: Basal GRK 2 levels are pending to be analyzed with a working antibody to GRK2. Statistically significant differences were identified between the study groups regarding IL1β production post-TLR2 stimulation. Namely, the FM group had a lower production of IL1β compared to controls (CON). TNFα measurements showed similar trends to IL1β in relation to study group designation but did not reach statistical significance. Both NFRT and pain area were negatively correlated with IL1β level post-TLR2 stimulation. Both TNFα and IL1β were positively correlated with the wind up ratio (WUR) measurement of CS at the dorsum of the foot.

Conclusion: In line with the hypotheses of the study, post-TLR2 stimulation in PBMCs there is a negative correlation between IL1β expression and NFRT. There is also a positive correlation of both TNFα and IL1β expression post-TLR2 stimulation on one hand, with Dorsum of foot WUR on the other. These correlations suggest that cytokines can be used as biomarkers for CS. However, and counter to the proposed hypotheses, Both FM and TMD study groups have higher NFRT values. In addition, IL1β expression post-TLR2 stimulation in PBMCs is significantly lower in the FM group compared to the CON group, while the TMD group was intermediate. This suggests that there might be an impaired TLR2 responsiveness associated with chronic pain

which could possibly be part of the mechanisms underlying CS in the spinal cord, considering the mechanistic similarities between PBMCs and glial cells in the contexts of pain and immunity.

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DEDICATION

I dedicate my thesis work to my wife, Maha, and my children, Abdullah and Leen, who ae my constant source of unconditional love and support.

Chapter 1. INTRODUCTION

1.1 CENTRAL SENSITIZATION AND CHRONIC PAIN DISORDERS.

Chronic pain disorders, such as Fibromyalgia (FM) and chronic temporomandibular disorders (TMD), exhibit common aspects including their challenging management and that there is an experience of persistent pain in the absence of obvious peripheral abnormalities (1-3).

FM is a common disease that manifests as diffuse, chronic pain of the musculoskeletal system. It affects 2–4% of adult females (9:1 female to male ratio) and is found in all ethnic groups, climates and cultures (4). Chronic widespread pain (CWP) is the fundamental feature of fibromyalgia (FM) which afflicts 10-15% of the world population (5). In terms of disability, the major symptoms that limit vocational tasks in FM patients are pain (87%), tiredness (80%), muscle weakness (73%), and memory and concentration problems (51%) (6). Children with FM are usually misdiagnosed and they can suffer from social isolation, malfunction and loss of school days, which directly and negatively impacts their education even through adulthood (7). A study of the economic burden of FM put the mean annual healthcare expenditures for FM patients at 10911 US dollars (SD = $16075), and the mean absence from work costs at ($3316, SD = $4763) (8).

Pain in the temporomandibular region is common as 10% of the population over age 18 have this pain condition (9). Patients with painful temporomandibular disorders have lower scores on most physical and social functioning items than the general population when evaluated for The Health Related Quality of Life (HRQoL) (10). Systematic reviews have found that there is an association between temporomandibular disorders and lower quality of life (11, 12).

The absence of peripheral abnormalities in theses prevalent disorders poses an interesting research question on the pathophysiological processes that cause them. The evidence so far points

to a dysregulation of pain processing in the central nervous system. This dysregulation is dubbed "Central Sensitization" (13, 14) and has been found in FM (15). FM displays spinal cord hyperexcitability which can cause enhanced pain from low intensity nociceptive or innocuous peripheral stimulation. This indicates the exaggerated activity of spinal pain pathways in these patients (15). In addition, the central endogenous pain inhibitory mechanisms in FM are dysfunctional as evidenced, for example, by the absence of modulation of pressure pain via heterotopic noxious conditioning stimulation (16), which contributes to amplified pain. Brain-imaging shows that brain regions involved in homeostatic control of pain are less connected in FM patients than healthy controls. This dysfunction might play a role in maintenance of FM pain (17). These central nervous system changes in FM are implicated in the exhibited hypersensitivity and allodynia.

Likewise, chronic TMD pain has also been linked to central sensitization (CS). Several studies demonstrated the TMD is associated with exaggerated pain sensitivity at extra-trigeminal body regions (14, 18). For example, pressure pain thresholds in extra-trigeminal areas are significantly lower in TMD subjects than in pain-free controls (19, 20). TMD is also linked to amplified responses to painful repeated stimuli of low intensity (21), and dysregulation of endogenous pain modulation (22). A 2017 systematic review and meta-analysis showed that TMD is associated with reduced pressure pain thresholds in both trigeminal and remote regions (23). In addition to CS, psychosocial factors such as stress, depression, and anxiety have been implicated as cofactors in both pain FM and TMD conditions (24-29).

These similarities between TMD and FM do not extend into their classification and diagnostic criteria: TMD is considered a regional pain syndrome, the region being orofacial (30). On the other

hand, as mentioned above, chronic widespread pain is the fundamental feature of fibromyalgia. To reach a preliminary diagnosis of FM, the American College of Rheumatology (ACR 2010) put forth the following three conditions (3):

1- Widespread Pain Index (WPI) is ≥ 7 and the Symptom Severity Score (SS) ≥ 5, or the WPI is 3–6 and the SS ≥ 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 (3).

In comparison, the Diagnostic Criteria of TMD (DC/TMD) (31) specifies a combination of symptoms, including pain, as well as clinical examination findings of the temporomandibular joint (TMJ) and masticatory musculature that control it in order to reach a diagnosis of specific subtypes of TMD.

FM and TMD are, of course, not mutually exclusive but frequently occur as co-morbidities (28, 32). TMD has a prevalence between 59.37% and 93.7% in FM patients (33), while in a study of 200 TMD patients, 163 patients described widespread pain in remote extra-trigeminal regions (34) and the prevalence of FM in TMD patients ranged from 10% to 52% (28, 32). Facial muscle pain occurs 31 times more often in FM patients than in those without FM (35), with FM patients often reporting myofascial pain (35, 36).

To summarize, FM and TMD share overlap in pain locations, a presumed pathophysiology, and contributing factors. The evidence pointing to CS as the underlying etiology of altered central pain processing in both disorders is growing more strongly and compelling.

1.2 MEASUREMENT OF CENTRAL SENSITIZATION

CS is challenging to evaluate clinically, especially if we wish to quantify its severity and extent. Hence, there is a need for a proper tool to do exactly these features (37). One such tool that has a long track record is Quantitative sensory testing (QST) (38), which tests for wind-up ratio (WUR), vibration detection threshold (VDT), tactile detection threshold (MDT), mechanical pain threshold (MPT), thermal detection and pain thresholds, mechanical pain sensitivity (MPS), dynamic mechanical allodynia (DMA), and pressure pain threshold (PPT) (39, 40), among other measures. As such, QST is non-invasive and measures both large and small nerve fiber function (Aβ, Aδ and C fiber), leading to the identification of the corresponding central pathway (41). It is used to assess neuropathic conditions and somatosensory profiles in non-neuropathic conditions such as myofascial pain and FM (42-45). QST holds enough reliability as a tool to be used to examine various pain disorders, whether trigeminal such as TMD or extra-trigeminal such as FM (39, 40, 46-48).

Another tool to assess CS is to measure the polysynaptic nociceptive flexion reflex (NFR), which objectively gauges spinal processes (49-51). NFR circuitry goes through Aδ and C nociceptive afferent fibers, interneurons, and alpha motor neurons (50). Experimentally, electrical current is used to elicit NFR, and the smallest electrical stimulations to initiate the reflex is the NFR threshold (NFRT), which correlates with subjective pain threshold (52-54). NFR has been used to gauge CS in several chronic pain disorders including FM (15, 51, 55-57).

Another reliable tool that evaluates CS in chronic pain disorders is by measuring Electrical Pain Threshold (EPT) (58, 59). When a non-pathological area displays pain hypersensitivity to electrical stimulation this would be an indication of dysregulated central processing (60).

Importantly, normative values for pain free individuals have been established for both NFRT and EPT (61).

1.3 IMMUNE REGULATION AND PERIPHERAL SENSITIZATION

The preclinical evidence that the immune system plays a major rule in the regulation of pain is compelling. For example, peripheral nociceptive neurons can be sensitized by the action of immune cells which release trophic factors and pro-inflammatory cytokines. (62).

Tissue injury elicits an action potential that travels centrally to the central terminal of the neuron and peripherally to the peripheral terminal of primary afferent neurons. The latter event results in a release of a number of signaling molecules such as substance P (SP), calcitonin gene-related peptides (CGRP), somatostatin and vasoactive intestinal peptide (VIP). These in turn increase vascular permeability and facilitates migration into the site of injury of immune cells such as neutrophils, mast cells, basophils and macrophages. Immune cells, in turn, release a number of inflammatory mediators such as leukotrienes, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα) (63). These inflammatory molecules then bind to receptors at the primary afferent's membrane, which triggers action potential firings. Some of these molecules also bind to G-protein coupled receptors (GPCRs), initiating a signaling cascade that changes the intracellular environment (64, 65). This decreases the thresholds of Aδ and C nociceptors, which in turn increases their sensitivity and excitability (63). Allodynia, and primary hyperalgesia (an exaggerated response to noxious stimuli at the location of tissue injury) are the result of this phenomenon designated as peripheral sensitization.

However, peripheral sensitization *per se* does not explain the symptomology of temporal summation of pain, dynamic tactile allodynia, and secondary hyperalgesia (pain initiated by

noxious stimuli away from the site of injury) which are seen in CS. The evidence thus far indicates that peripheral sensitization, propagated *via* C fiber nociceptors, can cause changes in the dorsal horn neurons of the spinal cord producing CS.

1.4 G-PROTEIN COUPLED RECEPTOR KINASE 2 IN CHRONIC PAIN

G-protein coupled receptor kinase 2 (GRK2) is an intracellular regulator of G protein-coupled receptors involved in homoeostasis through receptor desensitization (66). It also directly interacts with mitogen-activated protein kinases (MAPKs) signaling pathways which are part of the proinflammatory response (67). The action of GRK2 decreases the responsiveness of immune cells to cytokines, which protects the tissue from an excessive response to those endogenous proinflammatory molecules (68). In animal models of inflammatory and neuropathic pain, the downregulation of GRK2 resulted in CS and chronic hyperalgesia (66, 67, 69). In a rat model for trigeminal neuropathic pain with mechanical allodynia, there was a significant downregulation of neuronal GRK2 expression in the medullary dorsal horn. This allodynia could be reversed by blocking this downregulation (70). Thus, the evidence in hand suggests that the mechanism for long-term hyperalgesia involves lower levels of GRK2 expression or function.

One study found that GRK2 levels are higher in Peripheral Blood Monocytes (PBMCs) following experimental acute mental stress in healthy human subjects (71). This is interesting because chronic pain and chronic stress share significant physiological and conceptual overlaps (72).

1.5 CHRONIC PAIN AND GLIAL CELLS

Within the central nervous system (CNS) the activation of glial cells also releases trophic factors and pro-inflammatory cytokines which results in sensitization of central pain pathways

(62). Glial cells are non-neuronal cells endogenous to the central nervous system and include microglia, oligodendrocytes, and astrocytes; glial cells and have a major role in CNS homeostasis. For example, they control the microenvironment that surrounds neurons and support their communication. Glial cells also take up, metabolize, react to, and release neurotransmitters and other modulators (73). Microglia and astrocytes in particular act as the immune cells of the CNS and are activated under circumstances such as microbial invasion, injury of the CNS, and some pain states (74). Neuronal cells themselves under these circumstances release signaling molecules such as glutamate, BDNF, substance P, chemokines and ATP which activate neighboring glial cells. These in turn release cytokines such as IL1β, IL 6, and TNFα which act upon Aδ and C afferents fibers that synapse in the dorsal horn increasing even more their output of excitatory amino acids and substance P. The end-result is that dorsal horn neurons become hyperexcitable which causes CS (75).

Hence, maintenance of physiological homeostasis within both the peripheral and CNS components requires communication between neuronal and immune-non-neuronal cells. However, under pathophysiological circumstances, this communication can lead to neuro-disorders such as the shift from acute to chronic pain (76).

Animal studies implicate the activation of CNS glial cells in the initiation and maintenance of chronic pain (74, 77-83). These results cannot be confirmed in human subjects because it is not possible to sample and experiment on glial cells residing in the CNS of humans. Hence, it remains unclear whether the pathophysiological model of chronic pain gleaned from animal studies is actually applicable to humans (83).

1.6 Glial Cells and peripheral blood monocytes

Since it is not possible to study glial cells in human as we do in animal models, some studies have looked into Toll-Like Receptor (TLR) responsiveness in peripheral blood monocytes (PBMCs) as a surrogate for glial cells, with the assumption that TLRs and GRK2, which are found centrally in glial cells and in PBMCs (68, 69, 84), function similarly in CNS innate immune cells as in PBMCs. This approach is supported by two published studies that demonstrated that PBMCs from chronic pain sufferers, regardless of opioid use, had higher levels of IL1β expression than pain-free controls after being challenged with TLR agonists. The studies provided evidence that immune activation in PBMCs and in spinal cord were related to the pain state in a "dose"-related manner. These results indicate, not only a higher responsiveness of PBMCs in the presence of CS, but also suggest that PBMCs can potentially be used as a biomarker for chronic pain (85, 86). In PBMCs from patients with rheumatoid arthritis and multiple sclerosis, GRK2 levels are reduced, suggesting that measuring GRK2 in PBMCs is a potential biomarker for the endogenous control of inflammatory disease processes (87, 88).

1.7 Immune dysregulation and central sensitization

The role of immune dysregulation and CS has not been studied in chronic TMD patients. Several studies looked into the levels of cytokine production in FM patients (89-91). In a 2011 systematic review with meta-analysis looking into cytokines in fibromyalgia syndrome, the authors reported that some studies claimed a higher level of cytokine production in FM patients, while others had opposite results (90). In addition, several studies reported the absence of differences in cytokine levels between FM patients when compared to healthy controls (92-97). In FM patients, immune dysregulation has been reported in association with small-fiber neuropathy (98-100). However, the mechanisms that underpin interaction between the immune

system and pain modulation in FM remain elusive. One reason for this is the inconsistency of reports looking into production of cytokines. This inconsistency may be the result of research design. It is perhaps no surprise, then, that the authors of these systematic reviews have called for more studies that are hypothesis-driven and mechanistic (89, 90).

1.8 TOLL-LIKE RECEPTORS IN CHRONIC PAIN

Toll-like receptors (TLR) are cell-surface receptors that play a major role in innate immunity regulation in both peripheral and CNS components. Antigen presenting cells, such as monocytes and macrophages in the blood and other tissues, express TLRs (101). In the CNS microglia, astrocytes, oligodendrocytes and neurons also express these receptors (102).

TLR activation by endogenous damage-associated molecular patterns (DAMPs), or exogenous pathogen-associated molecular patterns (PAMPs) causes the release of pro-inflammatory cytokines. Exogenous PAMPs include lipopolysaccharide, single-stranded viral RNA and saturated fatty acid. Each of these are recognized by a specific TLR or TLR combination (84). There are ten functional TLR's identified in humans (TLR1–10) (103).

In models of nociceptive and neuropathic pain associated with pain hypersensitivity, TLR activation is a consistent finding (84) while blocking TLRs genetically or pharmacologically in animal models subdued the activation of microglia, which in turn reduced the production of pro-inflammatory cytokine and subsequent neuropathic pain (101).

A growing body of evidence from animal studies points to the dysregulation of innate immunity in the CNS as the underlying mechanism for chronic pain. The evidence supports a model where CS is a result of an exaggerated glial response through overproduction of pro-inflammatory mediators which in turn increases neuronal responsiveness (83).

1.9 Association between GRK 2 and toll-like receptors

The evidence points to an association between GRK2 and TLRs in which the action of TLR4 activation and subsequent IL1β release, causes suppression of GRK2 (104, 105). Importantly, it has been observed in PBMCs from patients with chronic pain that TLR stimulation increases the productions of IL1β from PBMCs above normal controls (85, 86). Thus, it is compelling to hypothesize that these molecular and functional changes we see in PBMCs, which are associated with pain hypersensitivity, have counterparts in innate immune components of glial cells in the CNS.

1.10 Pain and cytokines

Cytokines such as IL-1β, TNFα and IL-6 are produced following TLR activation in both glial cells and PBMC (84) and, as mentioned before, are believed to be part of the pathophysiology of peripheral sensitization (63) and CS (75). Cytokines also interact with other modulators of the immune response. For example, IL1β release, causes suppression of GRK2 (104, 105). In a rat model for trigeminal neuropathic pain with associated mechanical allodynia and downregulation of GRK2 (70), the medullary dorsal horn exhibited a colocalization of IL1β and its receptor in both the astrocytes and neurons, respectively. Blocking the receptor reversed the allodynia and the downregulation of GRK2. Chronic hyperalgesia is associated with increased TNFα in the spinal cord as measured in a mouse model that had a 50% reduction in GRK2 expression (67), suggesting that that in normal physiology, GRK2 inhibits overproduction of TNFα. The suppression of TNFα in the aforementioned mouse model reversed the hyperalgesia. Similarly, in a human subject study of GRK2 in PBMCs following acute mental stress, a significant negative correlation between GRK2 and TNFα levels was found across all study time points (71).

1.11 BUILDING UPON PREVIOUS WORK

This thesis is expanding on a previous pilot study that was entitled *G protein-coupled Receptor Kinase 2 (GRK2) and Toll-like Receptors as Regulators of Central Sensitization in Fibromyalgia and Chronic Temporomandibular Disorders* (106). I am using the data and samples collected from this previous study, which I will designate as "**pilot study-A**", and building upon it in my work.

Because of the strong evidence that implicates the immune system in modulating central pain pathways (107), pilot study-A examined the expression levels of GRK2, a negative modulator of the immune response, and the toll-like receptors' (TLR) downstream cytokine interleukin (IL)-1β, a positive modulator of the immune response. These two modulators are not independent from each other since IL1β signaling has been shown to reduce neuronal GRK2 expression (105). Importantly, both inflammatory modulators are found centrally in glial cells and in PBMCs (68, 84). Our ability to measure these molecules in PBMCs is clinically important because we can't measure them in glial cells of living human subjects. Interestingly, the protein levels of both have also been reported to be altered in PBMCs of patients with other types of chronic pain (85-87). In pilot study-A, the researcher successfully enrolled 11 TMD patients, 9 fibromyalgia (FM) patients and 18 controls to examine the levels of GRK2 and IL1β after TLR stimulation in blood samples from these three groups. The hypotheses of this study were: 1) PBMCs from FM and chronic TMD patients have lower basal GRK2 levels. 2) These levels are negatively correlated with indices of CS. 3) the blood of FM and TMD patients displays exaggerated IL1β production after TLRs stimulation. 4) IL1β levels correlate positively with indices of CS.

In a preliminary analysis of the recruited patients, pilot study-A exhibited a negative correlation between nociceptive reflex threshold and IL1β level following TLR stimulation, which was in line with one of the hypotheses of the study.

Chapter 2. AIMS AND HYPOTHESES

FM and TMD are both chronic pain syndromes that are challenging to manage, especially since they are not explained by tissue lesions (18). They both feature an increased activity of pain pathways at the central nervous system, i.e. CS (15, 18, 21). CS results in spontaneous activity of pain neurons, a larger distribution of receptive fields and an enhanced response to stimuli at the level of the spinal cord (108). The molecular mechanisms underlying CS are unclear, which hampers the development of targeted treatments.

With this thesis I worked to accomplish two aims:

1. Complete the goal of pilot study-A of recruiting patients to reach 18 subjects for the TMD and FM groups; to analyze both the levels of IL1β after TLR stimulation and basal GRK2 levels in all groups at $n = 18$; and test the hypotheses of the previous study.

2. Make use of the full complement of blood samples at $n = 18$ for each of the three study groups to expand on pilot study-A by measuring other molecular candidates of the immune system that I hypothesize to modulate CS. Specifically, I wished to look at the protein levels of tumor necrosis factor-alpha (TNFα) because it is downstream of TLR activation in both glial cells and PBMC (84), and is elevated in models of neuropathic pain (68).

Based on a previous study that detected a difference in GRK2 levels between 16 patients with rheumatoid arthritis and 16 healthy controls (87), I believe that my sample size is appropriate to detect existing differences between groups.

Hypotheses: The pro-inflammatory cytokines IL1β and TNFα are expected to be elevated in FM and TMD samples relative to controls, while the anti-inflammatory GRK2 is expected to be lower in FM and TMD samples relative to controls. With respect to indices of CS, I expected to find a positive correlation with pro-inflammatory cytokine expression and a negative correlation with GRK2 levels.

Chapter 3. MATERIALS AND METHODS

3.1 STUDY DESIGN AND OVERVIEW

The design of pilot study-A, which this thesis is expanding upon, was a case-control study, and as with the previous study (106) (details provided in *Appendices*), I continued recruiting female patients at the Center for Pain Relief and at the Oral Medicine Clinical Service of the University of Washington. I aimed to recruit an additional 7 TMD, 9 FM and no controls to reach an $n = 18$ for each of the three groups. As before, the diagnosis of FM was according to the revised criteria of the American College of Rheumatology (2) while the identifications of TMD was according to the Diagnostic Criteria for TMD (31). The methodology for patient selection, exclusion, recording of demographic and clinical characteristics, scoring (for jaw function, pain, depression and anxiety), measuring CS (which was quantified by pain reports to pressure, electrical and repeated mechanical stimulation, areas of pain, and withdrawal reflex of the leg) and statistical analysis is the same as pilot study-A (106) (details in Appendices A-F).

Measurement of GRK2 and TLR activation: GRK2 expression was not measured due to lack of working anti-GRK2 antibody at the time this thesis was written. However, it is to be measured, in future plans, in the blood samples collected as previously described (67, 87). In separate sample aliquots, I tested the responsiveness of PBMC to TLR stimulation by measuring IL1β and TNFα production (86). The following TLR agonists were added to the blood the stimulate cytokine production: the TLR2 agonist Pam3CYSK4, CL097 (TLR7/8 agonist), LPS (TLR4 agonist) and LPS with the NLRP3 agonist monosodium urate crystals (MSU) (details in Appendix G). Samples were analyzed for IL1β and TNFα using DuoSet ELISA Development kit (R&D system). The tests and blood drawn was performed 1-5 days after menstrual period began in order to account for possible variations in TLR activity and pain sensitivity (109, 110).

Statistical analysis: In addition to descriptive statistics (Percentages were calculated for discrete variables and means and standard deviations were computed for continuous variables), the primary analysis was a one-way-ANOVA with Bonferroni post-hoc testing and repeated measures ANOVA, with Group (controls vs. FM vs. TMD) as the between subject factor, and cytokine production levels post-TLR stimulation as the within subject factors. The dependent variables were post-TLR-stimulation cytokine concentration. I performed a Pearson product moment correlation between either GRK2 or cytokine levels on one hand and indices of CS on the other to examine correlations. A chi-square test was used to compare the racial composition of the three study groups. All analyses were performed using SPSS software (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp).

Chapter 4. RESULTS

This section is organized in the same fashion as the "Results" section from pilot study-A (106) to facilitate a better demonstration of the contribution that I added to the aforementioned study.

4.1 STUDY SAMPLE

Pilot study-A screened 24 individuals for the pain-free control (CON) research group, of whom 18 were eligible, enrolled and tested. The reasons for exclusion were tension-type headache, migraine or using systemic hormone replacement therapy. One subject from the CON group reported a migraine diagnosis after testing, and was excluded from the data analysis (106).

Pilot study-A screened 191 potential subjects for the TMD research group from whom 36 met the eligibility criteria. From these 36, 5 met the criteria for FM and were placed in that research group, 11 were enrolled and tested in the TMD group and the rest decided not to participate in the study. The reasons for exclusion were: "pain intensity less than 3 (n = 34), migraine diagnosis (n = 27), neurologic disease (n = 20), autoimmune disease (n = 17), opioid users. (n = 15), psychiatric disease other than anxiety and depression (n = 2), pregnancy (n = 1), use of systemic hormone contraceptives (n = 6), systemic hormone replacement therapy (n = 6), current infection (n = 3), and non-English speaker (n = 2)" (106).

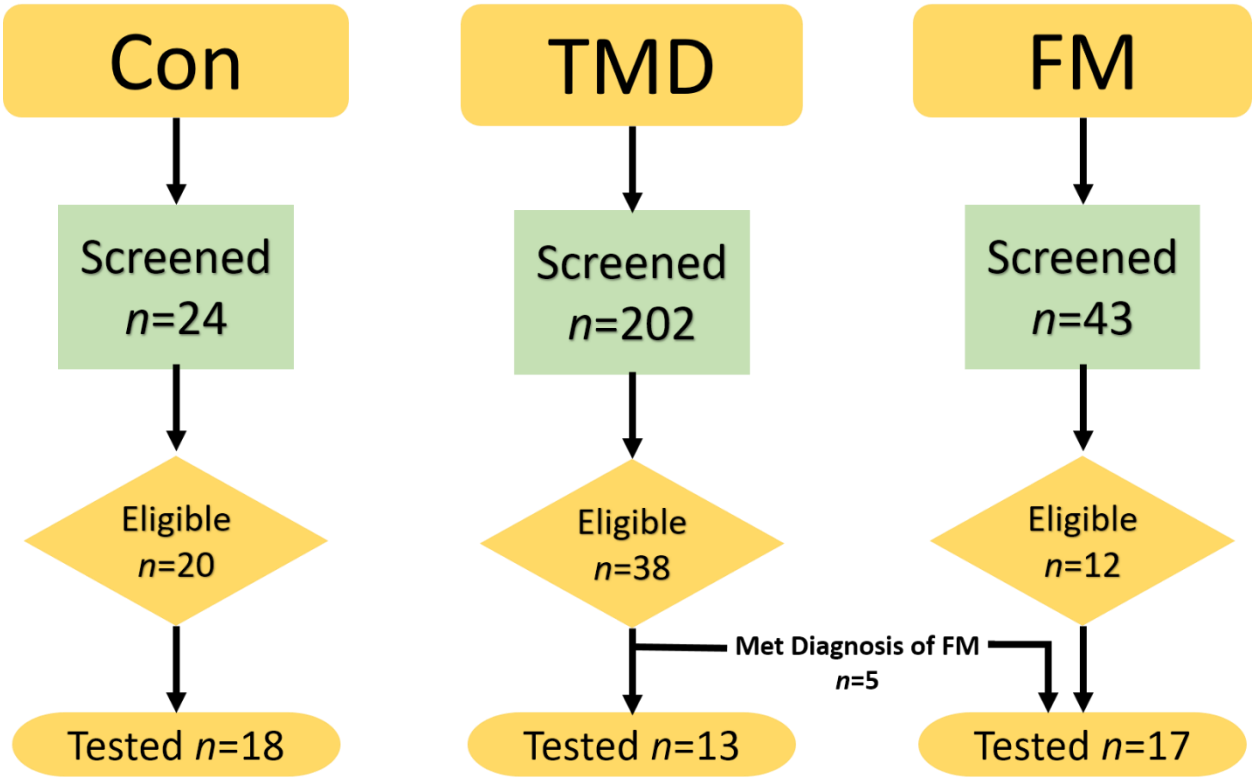

Figure 1. A flowchart demonstrating subject enrollment.

For the FM research group, 16 subjects were screened in pilot study-A, of whom 9 met the eligibility criteria and were enrolled and tested. The reasons for exclusion were opioid usage (n = 10) or autoimmune condition (n = 2).

I additionally added 2 subjects to the TMD research group and 8 subjects to the FM research group that were enrolled and tested. Fifteen individuals with FM were screened but not enrolled; the reasons for exclusion were: use of systemic hormone contraceptives (n = 6), pain intensity less than 3 (n = 1), migraine diagnosis (n = 2), neurologic disease (n = 2), opioid use (n = 2) and psychiatric disease other than anxiety and depression (n = 1). One subject pulled out of the study. Some of the excluded individuals had two or more disqualifying reasons.

Four individuals with both TMD and FM were screened but not enrolled; the reasons for exclusion were: use of systemic hormone contraceptives (n = 2) and opioid use (n = 2). A number of individuals were screened and found eligible for the FM or TMD research groups but could not be enrolled due to the restriction brought about by the COVID-19 pandemic.

Nine individuals with TMD were screened but not enrolled; the reasons for exclusion were: a diagnosis of endometriosis (n = 1), use of systemic hormone contraceptives (n = 4), pain intensity less than 3 (n = 3) and a migraine diagnosis (n = 1). Figure 1 summarizes the enrollment numbers of that are a combination from this study and pilot study-A.

4.2 Demographic, Pain-related, and Psychological data

Combining both my work and the work of pilot study-A (106), 18 subjects were enrolled and tested in the CON study group, 13 in TMD and in 17 FM. As mentioned before, one subject from the CON group reported a migraine diagnosis after testing, and was excluded from the data analysis. Demographic data, pain-related, and psychological variables are reported in Table 1.

The median age for the participants is not different across the three study groups. BMI, however, is significantly higher in the FM group compared to both the TMD and CON group ($p < 0.01$ with Bonferroni post-hoc correction). A Chi-square test also reveals significant difference in the racial composition of the three groups.

Regarding pain, I did not find any differences in pain duration, pain intensity or average pain between the TMD and FM study groups. There is a significant difference between TMD and FM groups in reported pain area ($p = 0.001$ after Bonferroni correction). From the *Pain interference* values, impact on *General activity*, *Falling asleep* and *Staying asleep* were significantly higher in the FM group compared to TMD according to the student t-test (Table 1).

One-way ANOVA analysis with Bonferroni corrections also reveals a significant difference in the Patient Health Questionnaire-9 (PHQ-9) depression scale across all three groups; FM being highest compared to CON ($p < 0.001$) then TMD ($p = 0.02$ with Bonferroni test). For the Generalized Anxiety Disorder-7 (GAD-7) anxiety scale, post-hoc analysis shows that FM scores are significantly higher than CON ($p < 0.001$). Post-hoc analysis for jaw functional limitation as measured by the Jaw Functional Limitation Scale-20 (JFLS-20) also reveals significant differences across all groups, being highest in the TMD group compared to CON as

expected ($p < 0.001$). The FM JFLS-20 values are also well above the CON group ($p = 0.004$) (Table 1).

Table 1 Demographic, pain-related and psychological variables.

	Con (n =17) Median(Q1-Q3)	TMD (n =12) Median(Q1-Q3)	FM (n =17) Median(Q1-Q3)	p -value
Age (years)	56 (39-62)	60 (38-65)	65 (52-70)	**0.68**
BMI (kg/m2)	24 (20-26)	23 (20-25)	28 (26-34)	**<0.001**
Race % (n)				
Asian/Pacific	41.2% (7)	8.3% (1)	5.9% (1)	
Hispanic	11.8% (2)	8.3% (1)	5.9% (1)	
Caucasian	29.4% (5)	83.3% (10)	52.9% (9)	**0.017**
African American	0	0	17.1% (3)	
American Indian/ Native Alaskan	0	0	5.9 % (1)	
≥2 identified ethnicities	17.6% (3)	0	11.8% (2)	
Area of pain (pixels)	0	76440 (27840, 114474) n =13	372156 (258907,887847)	**<0.001**
Pain duration (years)	0	4 (1,19)	12 (8,22)	0.4
Pain intensity at the testing day (0-10)	0	4 (3,5)	5 (4,6)	0.33
Average pain in the past week (0-10)	0	5 (3,6)	6 (5,7)	0.16
Pain interference (0-10)				
General activity	n/a	3 (2,6)	6 (5,7)	**0.025**
Enjoyment of life	n/a	4 (2,7)	6 (5,8)	0.24
Falling asleep	n/a	4 (3,5)	7 (3,8)	**0.028**
Staying asleep	n/a	4 (3,4)	6 (4,8)	**0.002**
PHQ-9	0 (0,1.5)	5 (3,8)	11 (5,12)	**<0.001**
GAD-7	0 (0,1)	1 (0, 6)	6 (1,10)	**<0.001**
JFLS-20	0 (0, 0)	61 (36, 92)	25 (9,64)	**<0.001**

Abbreviations

BMI: Body-mass index; PHQ-9: Patient Health Questionnaire-9; GAD-7: Generalized Anxiety Disorder-7; JFLS-20: Jaw Functional Limitation Scale-20; SD: standard deviation; Q1: first quartile; Q3 third quartile

4.3 Pain-related Measures

4.3.1 *Pressure Pain Threshold*

Analysis of PPT values with one-way ANOVA analysis and Bonferroni corrections reveals significant differences across the study group for the masseter and scapula sites, but not for the site of the second toe (See Figure 2 and Table 2). The post-hoc analysis shows that the masseter and scapula PPT values from TMD and FM groups are lower than those in the CON group (p = 0.001 and $p < 0.001$ respectively for masseter and $p < 0.001$ and $p = 0.004$ respectively for scapula). However, the PPT values for FM are not significantly different from those from the TMD group.

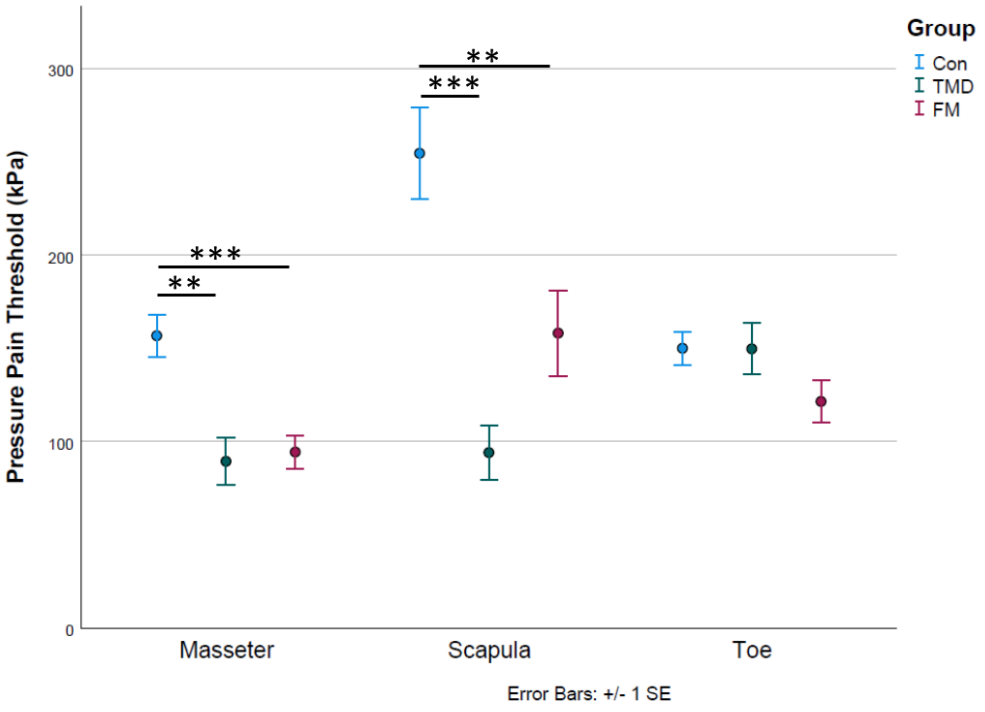

Figure 2. Mean pressure pain threshold (PPT) ± Standard error of the mean. ** denotes $p < 0.01$; ***$p < 0.001$

4.3.2 *Wind up ratio (WUR)*

A one-way ANOVA analysis of the WUR values for the masseter, scapula and dorsum of the foot sites, did not reveal any significant differences among the three study groups (See Figure 3. and Table 2. for summary statistics).

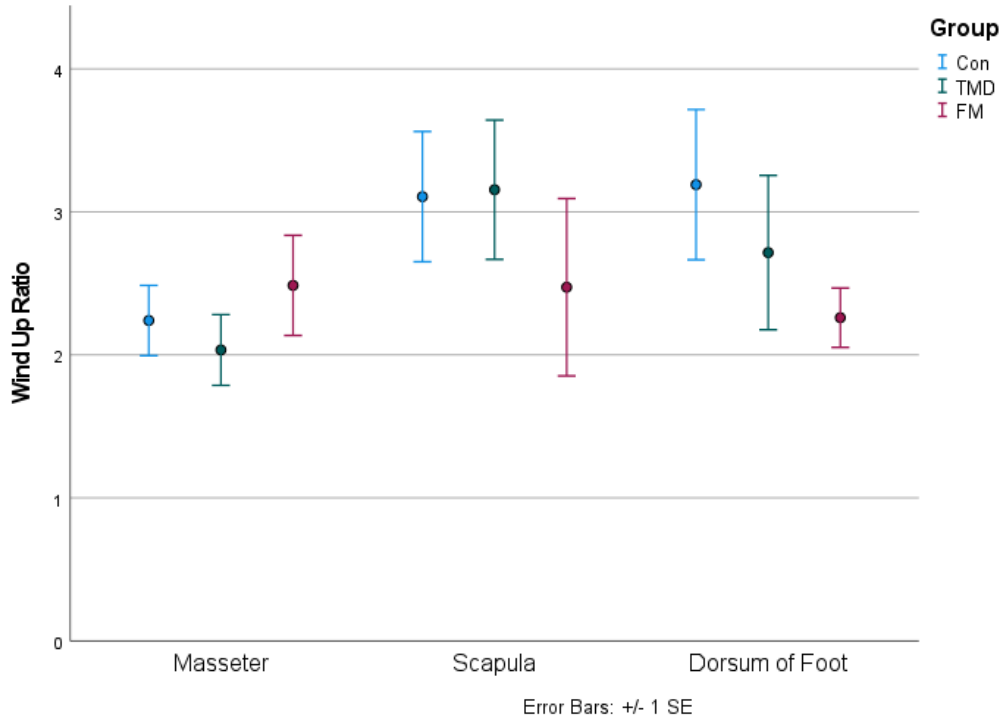

Figure 3. Mean wind up ratio (WUR) ± standard error of the mean.

4.3.3 *Nociceptive Reflex Threshold and Electrical Pain Threshold*

One-way ANOVA analysis of Nociceptive flexion reflex threshold (NFRT) values indicates significant differences across the three study groups ($p < 0.01$, see Figure 4). The Bonferroni post-hoc test reveals that both the TMD and FM groups have significantly higher NFRT values when compared to the CON group ($p < 0.05$). No statistical difference is found between the NFRT values of the TMD and FM groups.

One-way ANOVA of EPT values, indicates no statistically significant differences in electrical pain threshold among three groups ($p = 0.48$) (see Figure 5).

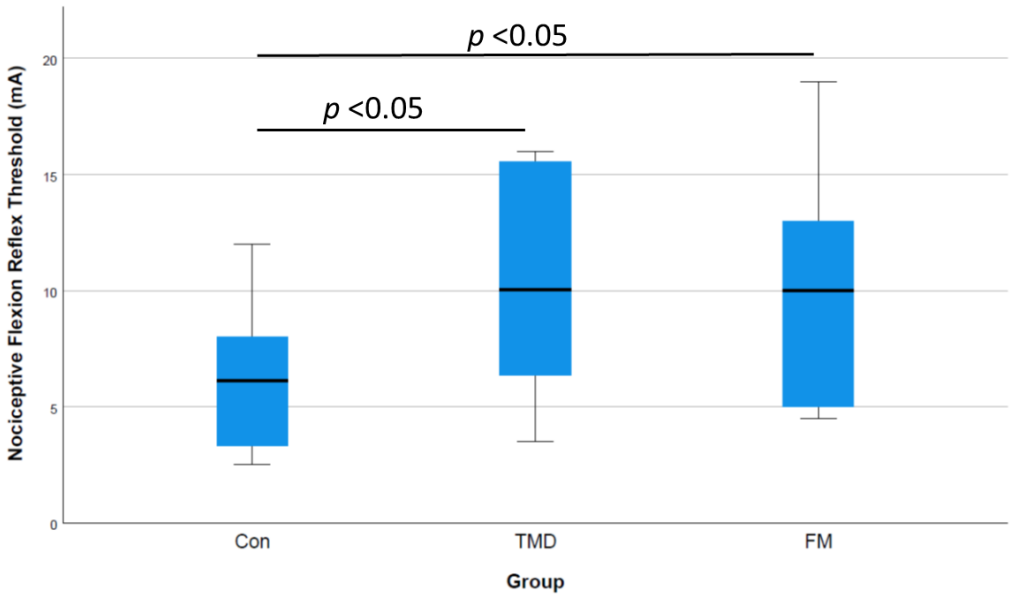

Figure 4. Nociceptive flexion reflex threshold in Controls, TMD patients, and FM

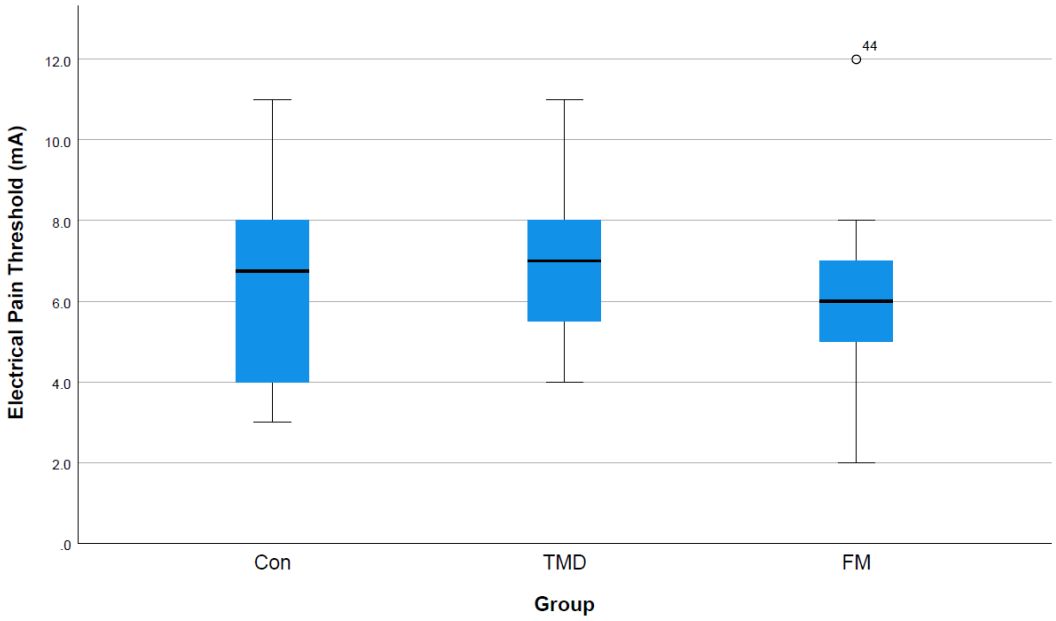

Figure 5. Electrical pain threshold (EPT) in controls, TMD patients, and FM patients.

Table 2. One-way ANOVA comparing pain-related measures between controls, patients with TMD, and patients with fibromyalgia.

	Con					TMD					FM					
	Mean	SD	Median	Q1	Q3	Mean	SD	Median	Q1	Q3	Mean	SD	Median	Q1	Q3	*p-Value*
PPT																
Toe	150	36	143	128	167	152	46	150	126	187	121	46	119	89	152	0.092
Masseter	159	45	150	133	190	97	50	85	62	129	94	37	96	68	113	**<0.0001**
Scapula	261	99	235	210	356	94	51	81	59	118	158	94	152	67	240	**<0.0001**
WUR																
Dorsum of foot	3.6	2.6	2.7	1.6	4.3	2.7	1.9	2.3	1.3	3.1	2.6	1.3	2.2	1.7	3.0	0.313
Masseter	2.2	1.0	1.9	1.4	2.8	2.1	0.9	1.8	1.5	2.7	2.6	1.3	2.3	1.7	3.6	0.410
Scapula	3.1	1.8	2.3	2.0	3.8	3.4	1.8	2.7	2.0	5.0	2.7	2.4	2.0	1.4	2.8	0.681
NFRT (mA)	6.0	2.8	6.1	3.3	8.0	10.4	4.7	10.0	6.4	15.6	10.1	4.9	10.0	5.0	13.0	**0.010**
EPT (mA)	6.5	2.7	6.8	4.0	8.0	7.2	2.2	7.0	5.5	8.0	6.0	2.4	6.0	5.0	7.0	0.480
Pain area (pixel)	0	0	0	0	0	70144	48566	76440	27840	114474	605417	512587	348986	251148	849412	**<0.0001**

Abbreviations

PPT: Pressure pain threshold; Toe: second toe; WUR: wind up ratio; NFRT: nociceptive reflex threshold; EPT: electrical pain threshold; SD: standard deviation; Q1: first quartile; Q3 third quartile

4.3.4 *Anatomical Distribution of Pain*

Bonferroni post-hoc testing indicates a significantly larger pain area in the FM group compared to both the CON and TMD group ($p < 0.001$). The TMD group, however, is not significantly different from the CON group (Figure 6).

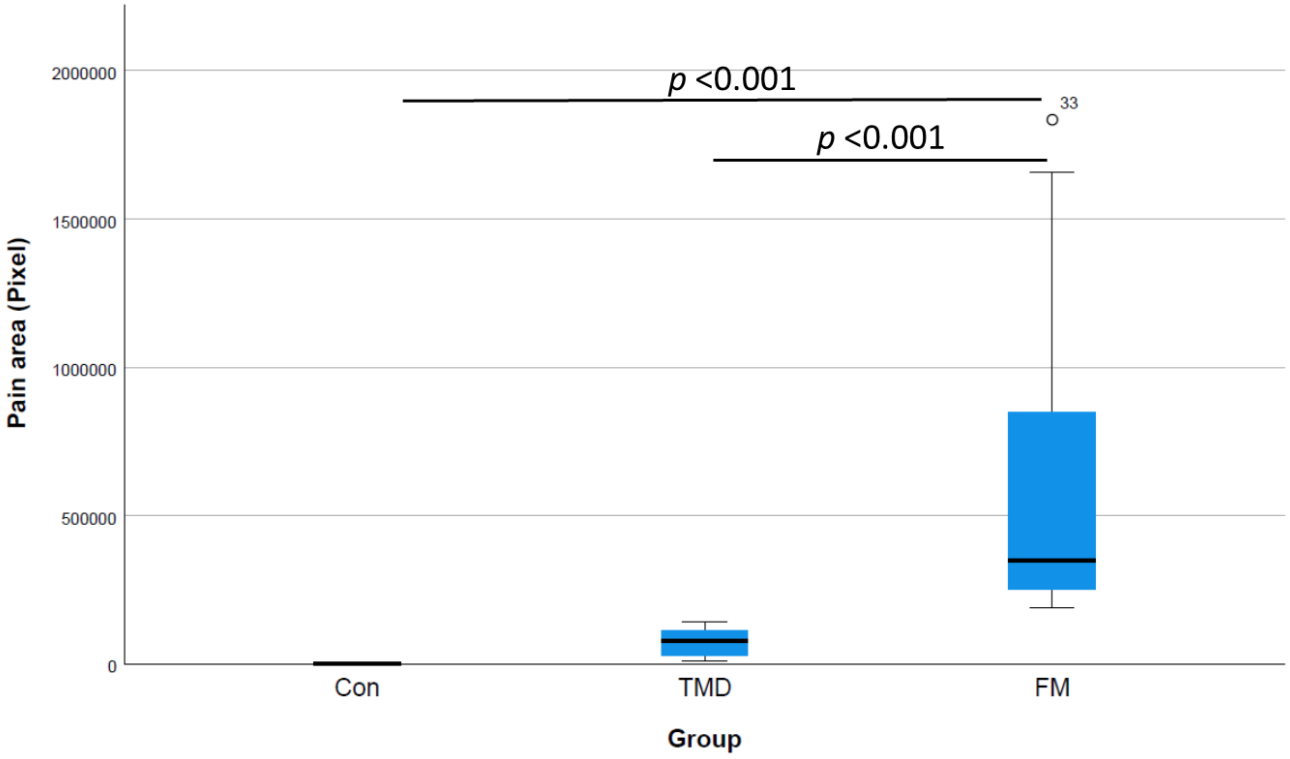

Figure 6. Pain area in controls, TMD patients, and FM patients.

4.4 BASAL GRK 2 LEVEL

I am currently conducting the western blot analysis to evaluate GRK 2 level from all participants. The results, therefore, will be reported in the future.

4.5 IL1-BETA LEVEL FOLLOWING TLR2 STIMULATION

The TLR2 agonist (Pam3CYSK4) was used to stimulate the blood collected from the research subjects at 10 ng/ml, 100ng/ml and 1000 ng/ml. One-way ANOVA analysis (Table 3.1) shows that there is a significant difference among the three study groups in IL1β expression at 100- and 1000 ng/ml of agonist concentrations ($p = 0.008$ and $p = 0.003$, respectively) (Table 3.1). Repeated measures ANOVA analyzing for interactions between all levels of Pam3CYSK4 concentration, and group designation (CON, TMD, FM), reveals a statistically significant difference (Table 3.2).

A post-hoc analysis indicates that the FM group has a significantly reduced IL1β production compared to the CON group. This post-hoc test was done with either the Bonferroni method ($p = 0.002$), or the Games–Howell method ($p = 0.003$). The TMD group is in between the FM and CON groups in IL1β expression but IL1β levels in TMD patients are not statistically significantly different when compared to either the CON group (Pairwise comparison $p = 0.12$) or the FM group (Pairwise comparison $p = 0.07$).

Table 3.1 Changes of IL1β level after TLR2 stimulation

Changes in IL1β level after TLR 2 agonist stimulation (pg/ml)

Agonist dose		10 ng/ml						100 ng/ml						1000ng/ml					
		Mean	SD	Median	Q1	Q3	*p*-value	Mean	SD	Median	Q1	Q3	*p*-value	Mean	SD	Median	Q1	Q3	*p*-value
Group	Con	39.4	34.3	34.3	10.4	50.2	0.27	103.8	64.7	111.6	35.6	144.6	0.008	195.1	133.5	180.2	67.7	308.4	0.003
	TMD	27.3	37.7	14.2	4.3	34.2		84.6	85.9	61.5	28.6	119.1		120.2	107.4	89.2	40.5	184.8	
	FM	17.2	41.1	6.2	0.2	12.4		32.3	30.2	22.1	10.5	50.0		59.4	49.5	44.9	24.7	105.9	

Table 3.2 Test of Between-subjects effects (Post-Pam3CYSK4 IL1β levels).

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	*p* -value
Intercept	746412	1	746412	72.47	<0.0001
Group	136348	2	68174	6.62	**0.003**
Error	422263	41	10299		

4.6 CORRELATIONS BETWEEN CENTRAL SENSITIZATION MEASURES AND IL1-BETA LEVELS

In Table 4, we observe a significant negative correlation between IL1β concentration post-TLR2 agonist stimulation (Pam3CYSK4 1000 ng/ml) on one hand, and NFRT (r = -0.370, *P* = .022) and *Pain area* (r = -0.311, *p* = 0.042) on the other. There is also a positive correlation with *Dorsum of foot* WUR values (r = 0.353, *p* = 0.022). Figures 7.1 to 7.3 illustrate these correlations.

No significant correlations between IL1β levels (released after 1000 ng/ml of Pam3CYSK4) and other QST measures were identified. Detailed correlations and *p*-values are shown in Table 4.

At a lower concentration of this TLR2 agonist (100 ng/ml), the negative correlation of IL1β levels with NFRT observed above loses its statistical significance but is trending in the same direction (Pearson correlation = -0.279, *p* = 0.09). *Dorsum of Foot* WUR and *Pain area* correlations with IL1β levels are in the same direction seen with the higher dose of TLR2 agonist and are statistically significant (Pearson correlation 0.399, *p* = 0.009 and Pearson correlation = -0.316, *p* = 0.039 respectively).

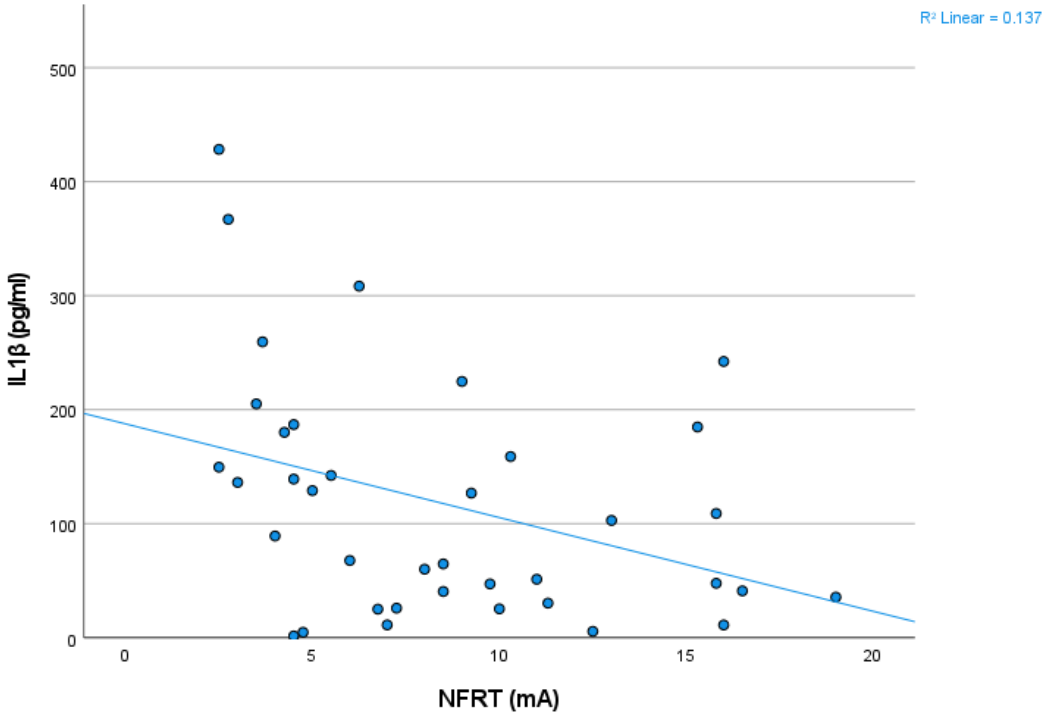

Figure 7.1 Scatter plots illustrating the relationship between IL1β level and the nociceptive flexion reflex threshold (NFRT).

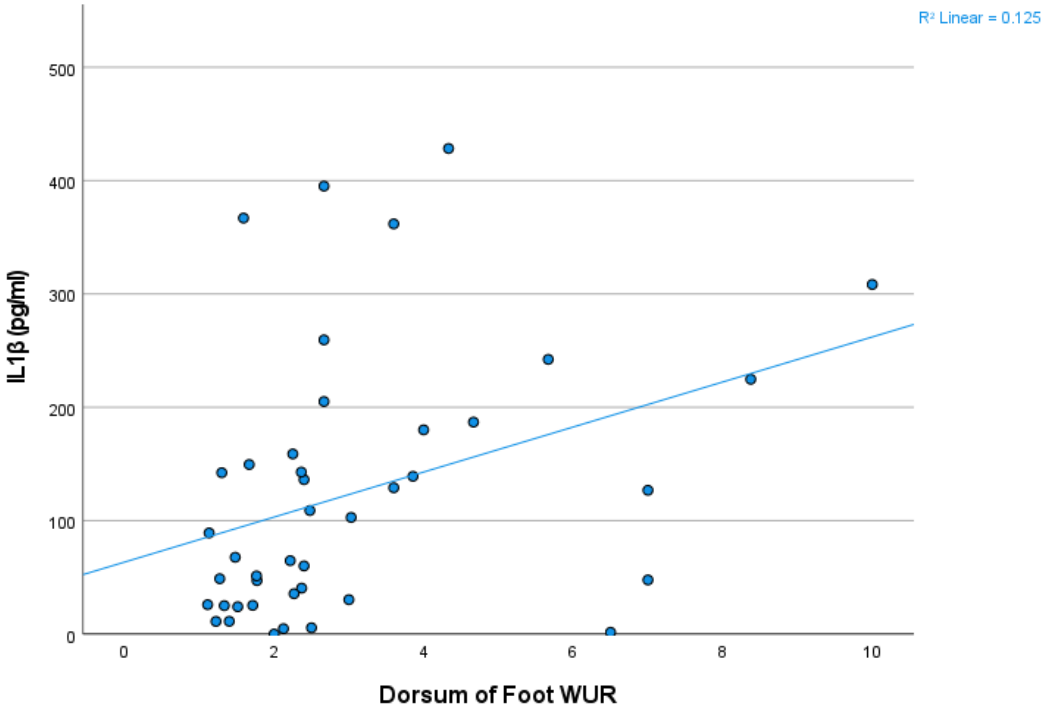

Figure 7.2 Scatter plots illustrating the relationship between IL1β level and Dorsum of foot wind up ratio (WUR).

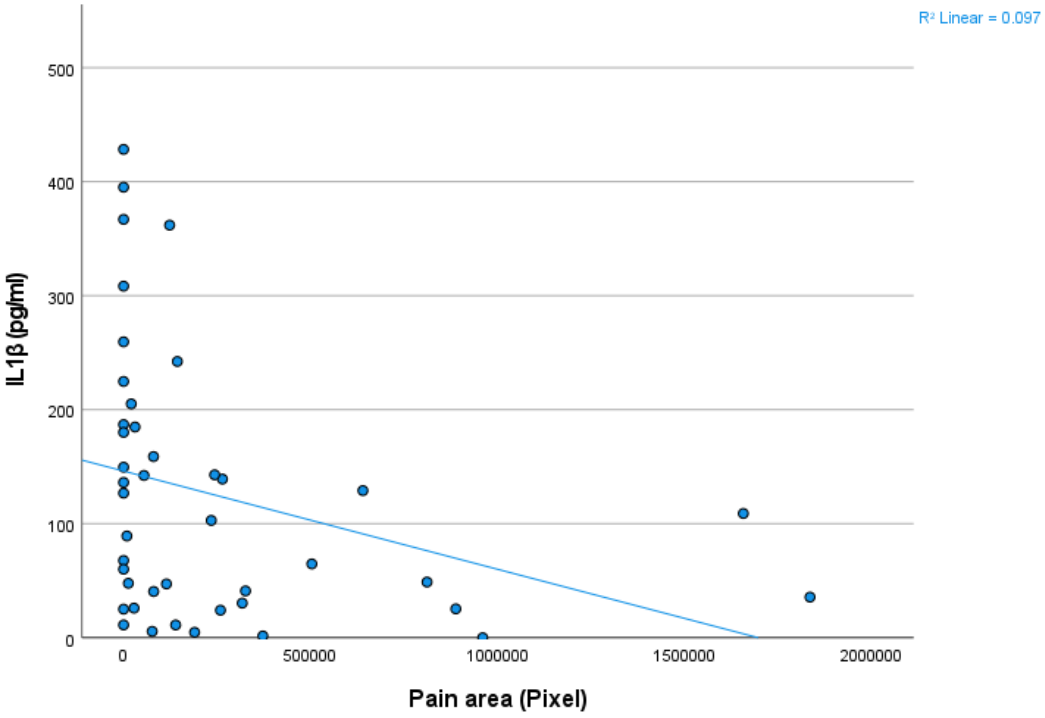

Figure 7.3 Scatter plots illustrating the relationship between IL1β level and pain area.

Table 4. Pearson correlation between measures of CS and IL1β expression after TLR2 stimulation (by Pam3CYSK4 1000ng/ml)

Pearson Correlations		
CS measure	**Correlation with IL1β level**	***p* -value***
Toe PPT (kPa)	0.027	0.86
Masseter PPT (kPa)	0.184	0.23
Scapula PPT (kPa)	0.205	0.19
Dorsum of Foot WUR	0.353	**0.022**
Masseter WUR	0.048	0.76
Scapula WUR	0.104	0.51
NFRT (mA)	-0.37	**0.022**
EPT (mA)	0.051	0.75
Pain area (Pixel)	-0.311	**0.042**
*Correlation is significant at the 0.05 level (2-tailed).		

Abbreviations
CS: Central sensitization
PPT: Pressure pain threshold
(Kilopascal, kPa)
WUR: Wind up ratio
Pixel: numbers of pixels

4.7 TNF-ALPHA LEVEL FOLLOWING TLR2 STIMULATION

I quantified TNFα levels from blood collected from research subjects where the TLR2 agonist (Pam3CYSK4) was used to stimulate it at 10 ng/ml, 100ng/ml and 1000 ng/ml. One-way ANOVA analysis shows that there are no significant differences between the three study groups in TNFα levels at 10- 100- or 1000 ng/ml of agonist concentrations (Table 5.1). Repeated measures ANOVA analyzing for interactions between all levels of Pam3CYSK4 concentration, and group designation (CON, TMD, FM), indicates no statistically significant difference between the study groups (Table 5.2).

Table 5.1 Changes of TNFα level after TLR2 stimulation

Changes in TNFα level after TLR 2 agonist stimulation (pg/ml)

Agonist dose		10 ng/ml						100 ng/ml						1000ng/ml					
		Mean	SD	Median	Q1	Q3	*p*-value	Mean	SD	Median	Q1	Q3	*p*-value	Mean	SD	Median	Q1	Q3	*p*-value
Group	Con	40.0	50.8	20.0	0.0	60.3	0.91	90.2	75.8	78.3	35.8	164.0	0.57	228.4	155.6	255.0	114.1	329.6	0.15
	TMD	30.5	50.4	0.0	0.0	37.3		77.3	60.0	74.8	26.9	116.0		146.6	98.0	177.5	75.8	213.3	
	FM	36.8	71.9	0.0	0.0	27.8		62.9	78.8	32.4	0.0	85.7		133.1	159.2	86.7	0.0	194.5	

Table 5.2 Test of within-subjects effects (Post-Pam3CYSK4 TNFα levels).

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	*p* -value
Intercept	1177647	1	1177647	57.1	<0.001
Group	46312	2	23156	1.12	0.34
Error	866392	42	20628		

4.8 CORRELATIONS BETWEEN CENTRAL SENSITIZATION MEASURES AND TNF-ALPHA LEVELS

In Table 6, we observe a significant positive correlation between TNFα concentration post-TLR2 agonist stimulation (Pam3CYSK4 1000 ng/ml) and *Dorsum of Foot* WUR (r = 0.355, p = 0.02). Figure 8 illustrates this correlation. No other significant correlation with the CS measures is observed.

Table 6. Pearson correlation between measures of CS and TNFα expression after TLR2 stimulation (by Pam3CYSK4 1000ng/ml)

Pearson Correlations		
CS measure	**Correlation with TNFα level**	***p* -value***
Toe PPT (kPa)	0.161	0.3
Masseter PPT (kPa)	0.101	0.51
Scapula PPT (kPa)	0.114	0.46
Dorsum of Foot WUR	0.355	**0.02**
Masseter WUR	0.000	1
Scapula WUR	-0.126	0.43
NFRT (mA)	-0.261	0.11
EPT (mA)	-0.006	0.97
Pain area (Pixel)	-0.187	0.23
*Correlation is significant at the 0.05 level (2-tailed).		

Abbreviations
CS: Central sensitization
PPT: Pressure pain threshold (Kilopascal, kPa)
WUR: Wind up ratio
Pixel: numbers of pixels

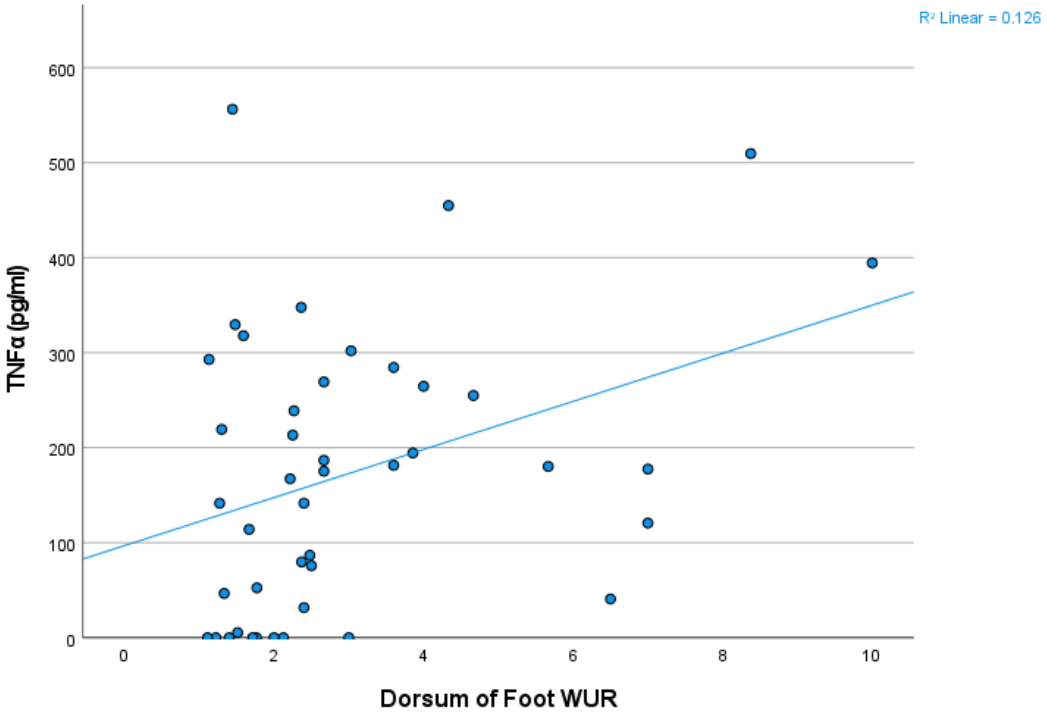

Figure 8. Scatter plots illustrating the relationship between TNFα level and Dorsum of foot WUR.

4.9 Analysis of stimulation by Other TLR agonists

In addition to the TLR2 agonist Pam3CYSK4, we also used CL097 (TLR7/8 agonist), LPS (TLR4 agonist) and LPS combined with the NLRP3-agonist monosodium urate crystals (MSU) to stimulate cytokine production from PBMCs.

All of these agonists elicited TNFα and IL1β production in a dose-dependent manner (not shown). However, a one-way ANOVA analysis on the effect of the above agonists did not reveal any statistically significant differences among the three study groups in post-TLR stimulation cytokine levels at any of the specified doses.

Examining the effect of different doses of agonist on the three study groups using the repeated measures ANOVA test also failed to show any significant differences in cytokine levels among the groups.

Chapter 5. DISCUSSION

5.1 PAIN RELATED MEASURES

5.1.1 *Pressure pain threshold*

In line with previous published work (18, 23, 111, 112) and with the previous pilot study-A (106), PPT over the masseter and scapula was significantly lower in the TMD and FM groups compared to the CON group. However, no differences were seen between TMD and FM in any of the tested regions, even though individuals with both conditions were allocated to the FM group. This could be used as evidence that the level of CS in FM and TMD individuals may be similar even though the area of reported pain is very different in terms of location and total area.

5.1.2 *Wind up ratio*

The WUR test failed to show significant differences among the three groups. This could be due to the relatively small sample size and that two operators performed the test; myself and the researcher from pilot study-A (106). In a study that looked into test-retest reliability (TR-R) and the interobserver reliability (IO-R) of different QST measurements in patients with sensory disturbances of different etiologies, it was found that WUR had a mild correlation (TR-R: r=0.67; IO-R: r=0.56) (113). The reliability of the WUR test is reported to be higher in chronic orofacial pain patients (40). In this study, many research subjects had difficulty rating the pain using a 0-100 scale. Some chose to rate pain within

0-10, which could contribute to increased variance in the data that would obscure statistically significant differences.

Interestingly though, when the entire study cohort regardless of group was analyzed for correlations, I found a significant positive correlation between *Dorsum of foot* WUR on one hand and the production of either TNFα or IL1β on the other. This correlation at a larger n-value suggests that a larger sample is needed for the analysis of WUR measurements when a difference between experimental conditions is hypothesized.

5.1.3 *Nociceptive flexion reflex threshold*

My addition of 8 FM subjects did not change the finding from the previous work that NFRT in the FM group is significantly higher when compared to the control group (106). This result contradicts other CS studies that report a reduced NFRT (15, 114), and a very recent study that reports no changes in NFRT in FM patients (115). However, in this latter study when subjective pain ratings were plotted against the NFRT values of FM patients and healthy controls, only in FM patients was there a positive linear correlation seen (Spearman's Rho = 0.460; $p = 0.012$). This is very similar to the positive correlation I see between *Pain area* and NFRT in the FM group (Pearson correlation = 0.629; $p = 0.028$). For several of the research subjects in the FM group, it was not possible to determine the NFRT. This is consistent with studies that found that NFRT might be impaired by depression and fibromyalgia, due to desensitization of NFR pathways (116, 117). This higher NFRT in the FM group could also be a result of consuming drugs that modulate pain such as acetaminophen, NSAIDs, muscle relaxants, and antidepressants, as

reported by published studies (118-120). However, I did not investigate this possibility in this study.

My addition of 2 TMD subjects did not change the finding from the previous work that the TMD group have a significantly higher value for NFRT than controls. This finding is similar to the FM group and might be due to the same mechanisms.

5.1.4 *Electrical pain threshold*

In line with previous published work and with the pilot study-A (106), there was no significant difference among the study groups in EPT values, which could be due to the small sample size. Previous studies using QST, including EPT, suggest that EPT is not a very sensitive tool to measure CS (15, 121).

5.1.5 *Area of pain*

As expected, *Pain area* was significantly different among the three study groups, being highest in FM group, followed by TMD. The CON group, of course was pain-free. It is interesting to note that a proportion of FM participants also had TMD. This is consistent with reports that these two conditions commonly coexist (28, 32, 33, 35, 36).

5.2 IL1-BETA & TNF-ALPHA LEVELS FOLLOWING TLR2 STIMULATION

The FM group had a significantly reduced IL1β production compared to the CON group post-TLR2 stimulation. The TMD group was also trending in the same direction but did not reach significance. These findings indicate impaired TLR2 responsiveness in our chronic pain groups. This is contradictory to published clinical studies from Kwok et. al., which demonstrated that IL1β expression was significantly higher in chronic pain patients after TLR2

stimulation relative to pain free controls (85, 86). Those studies, however, had more diverse chronic pain populations. A systematic review with meta-analysis looking into cytokines in fibromyalgia syndrome, reported that some studies claimed a higher level of cytokine production in FM patients, while others had opposite results, (90) as we observe in our FM group. Although there were not statistically significant differences in age across the participant groups, more postmenopausal women were tested in the TMD and FM group than in the CON group. Pilot study-A reported that *seven out of 17 controls were premenopausal. Three out of 11 TMD subjects were of reproductive age. Of those women who were of reproductive age, one person did not experience menstruation due to ovariectomies. Two out of 9 FM subjects still experienced regular menstruation* (106). From the eight subjects I added to the FM group, 6 were postmenopausal and one was perimenopausal with sporadic, infrequent menstrual periods. The two subjects I added to the TMD group had regular periods. Postmenopausal women have chronically high levels of pro-inflammatory cytokines and a diminished ability to respond to pathogens or stimuli since the absence of estrogen with aging results in loss of TLR function (122). This could explain the significantly reduced response to TLR2 stimulation in the FM and TMD groups.

The TNFα levels were not statistically different across groups but were trending to be lower in the FM and TMD groups compared to control. This is similar to what I observe with IL1β expression levels and might be caused by the same mechanisms.

The levels of IL1β and TNFα downstream of other TLR- agonist stimulation (TLR4 and TLR7/8) were not statistically different among the three study groups. This could indicate that the TLR impairment observed in our chronic pain groups is specific to TLR2. The studies from Kwok et. al showed increased levels of IL1β, not just post-TLR2 stimulation, but also post-TLR4

and -TLR7 stimulation (85, 86). However, from the nineteen chronic pain patients in that study, only two had FM and none had TMD. Most had a diagnosis of joint pain (six had osteoarthritis, and six had back pain). Hence, the differences in TLR responsiveness between this study and the published work by Kwok et. al could be because of the very different populations of chronic pain subjects studied.

Most studies, when examining TLR2 signaling, show that the common immune response measured by function is an increase in IL-6 and TNFα production during acute processes, and IL-10 in chronic infections (123). This study did look at TNFα levels post-TLR2 stimulation and it would be of interest to examine whether IL-6 and IL-10 show similar trends in our sample.

5.3 Correlations between IL1B, TNF-alpha and clinical pain measures

Regarding the indices of CS, I found a negative correlation between NFRT, on one hand, the and level of IL1β release post-TLR2 stimulation on the other. There was also a positive correlation between *Dorsum of foot* WUR and both TNFα and IL1β release. This suggests that IL1β and TNFα expression downstream of TLR2 responsiveness can potentially serve as biomarkers for CS. A larger study would be needed to establish diagnostic thresholds, if any, linking IL1β and TNFα levels to CS in FM or TMD.

However, there is also a negative correlation between *Pain area* and level of IL1β release post-TLR2 stimulation, which is to be expected since the group with the largest pain area is also the group with the lowest level of IL1β release post-TLR2 stimulation; i.e. the FM group. It is also of interest to note that the *Dorsum of foot* WUR is not statistically different among the three study groups. There seems to be a disconnect between the statistical correlation between cytokine production and CS indices, and the finding that the FM and TMD groups have lower cytokine levels than CON. This disconnect is confounded further by the finding that NFRT is higher in

FM and TMD relative to CON. This could be explained by that the correlation analysis examined the entire study cohort regardless of group which allowed these correlations to surface at this higher *n*-value.

5.4 STUDY STRENGTHS AND LIMITATIONS

This and pilot study-A (106) are the first work to examine the role of the immune response and its relationship to CS in fibromyalgia and chronic TMD. This work controlled for several confounders, through strict exclusion criteria, that can potentially change the responsiveness of TLRs and CS measures (sex, age, autoimmune diseases, neurologic diseases, migraine, opioid use, systemic sex hormone therapy, etc.).

The limitations of this study include: (A) the small sample size; (B) We did not control for the racial composition of the study groups which are significantly different, nor did we control for BMI which is higher in the FM group. Both obesity and race can impact the immune system (124, 125) and subsequently affect our measurements; (C) time of blood draw was not uniform across subjects. This could potentially have affected leukocyte response due to the physiologic circadian rhythm since many immune parameters exhibit systematic fluctuations in human blood over the 24-h day (126); (D) Other than opioid usage, we did not control for pain-related medications such as NSAIDs, analgesics, SSRIs or SNRIs, some of which are reported to effect TLR expression and some pain-related measures (127, 128); (E) Inter-operator variability: In the previous study (106), the researcher had the responsibility for recruiting and screening potential subjects and performing QST, while another (a laboratory technician) had the responsibility of stimulating the collected blood with TLR agonists, isolating PBMCs to makes a protein lysate, and performing the ELISA and Western blot assays to quantify the proteins of interest. The previous study also had a volunteer who had helped with recruitment and screening, and had the responsibility of escorting the subject

to the phlebotomist to collect blood which she then delivered to the aforementioned lab technician at a site that is 30 minutes away. In this study I was responsible for performing all of these tasks. I was trained by the previous researcher on collecting QST measures and followed all the protocols from the lab technician, but variability in technique and differences in lab reagent characteristics from one lot to another or due to "aging" of reagents will inevitably increase variability in the data.

5.5 Conclusion

In line with the hypotheses of the study, PPT is lower in FM and TMD subjects. Correlation analysis for indices of CS with IL1β expression, post-TLR2 stimulation in PBMCs, revealed a negative correlation with NFRT. There is also a positive correlation of both TNFα and IL1β expression post-TLR2 stimulation on one hand, with *Dorsum of foot* WUR on the other. These correlations, if established in a larger sample size, would suggest that these cytokines can be used as biomarkers for CS.

However, and counter to the proposed hypotheses, both FM and TMD study groups have higher NFRT values. In addition, IL1β expression post-TLR2 stimulation in PBMCs is significantly lower in the FM group compared to the CON group, while the TMD group was intermediate. This suggests that there might be an impaired TLR2 responsiveness associated with chronic pain which could possibly be part of the mechanisms underlying CS in the spinal cord, considering the mechanistic similarities between PBMCs and glial cells in the contexts of pain and immunity.

Even though this is a pilot study with a small sample size, it reveals novel, statistically significant differences and interesting trends that warrant further investigation into the role of TLR2, IL1β and TNFα in CS in a larger sample. It also provides a basis to investigate a wider array of immune modulators in the context of chronic pain conditions, especially TMD and FM.

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APPENDICES

A. UNIVERSITY OF WASHINGTON CONSENT FORM

UNIVERSITY OF WASHINGTON
CONSENT FORM

G protein-coupled receptor kinase 2 (GRK2) as a regulator of central sensitization in fibromyalgia and chronic temporomandibular disorder (TMD)

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Researchers' statement

We are asking you to be in a research study. The purpose of this consent form is to give you the information you will need to help you decide whether to be in the study or not. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions, you can decide if you want to be in the study or not. This process is called "informed consent." We will give you a copy of this form for your records.

PURPOSE OF THE STUDY

The University of Washington, Department of Anesthesiology & Pain Medicine in collaboration with the Department of Oral Medicine is conducting a study to find out more about pain sensitivity in individuals with Fibromyalgia or temporomandibular disorder (TMD).

STUDY PROCEDURES

If you decide to participate in this research study, there will be one or two visits lasting up to 2 hours total. The study visit(s) will include a pain screening, questionnaires, pain sensitivity tests, and a blood test. During the pain sensitivity tests, you will be seated in a comfortable upright position in a quiet room. The tests will be performed on the painful side of TMD subjects and the dominant side of FM and control subjects.

Study Visit(s) Procedures:

1. Take a urine pregnancy test if of reproductive age (5 minutes).
2. Study questionnaires (30 minutes): We will record age, ethnicity, body-mass index, details of your pain (FM and/or TMD), duration of pain, pain medication, current pain intensity, average pain intensity during last month, depression assessment, anxiety assessment
3. Pain sensitivity will be assessed by:
    a. Referred Pain Area (5 minutes): using a personal tablet, you will draw where your pain in located on a 3D body image using a stylus pen. This functions like a pen on paper but the researcher is able to extract your exact area of pain using a specialized application.
    b. Pressure (10 minutes): This is a test that will be done in 2 locations, at the center of your 2nd toe, and for FM: most painful spot of your shoulder blade or for TMD: most painful spot of the rear cheek. In each location, pressure is applied using a small probe, the size of a sugar cube. This pressure will feel like someone is pressing down on your toe or shoulder blade or cheek. The pressure is increased until you feel that the pressure has

become painful. You will be instructed to press a button when it reaches that point and the test will stop. This test is repeated three times (at each location).

c. Electrical Stimulation (10 minutes): Electromyography (EMG) evaluates and records electrical activity produced by skeletal muscles. EMG is performed using an instrument called an electromyograph. Electrical stimulation will be performed through two electrodes placed on the top and bottom of your foot and the response will be recorded at 3 locations on your shin (front part of your lower leg). The current intensity will be increased until a reflex sensation (wanting to pull your foot away) and pain sensation is detected.

d. Windup ratio (5 minutes): Windup ratio is a test of repeated touches of a small metal rod touched to your skin. A single touch is made and you will rate the sensation on a 0 (no pain)-100 (worst pain imaginable) scale. After 10 seconds, 10 more touches are made and you will rate the sensation after the 10th touch. This will be done at 2 locations: the top of your foot, and for FM: most painful spot on your shoulder blade and for TMD: the most painful spot of the rear cheek.

e. Dynamic Mechanical Allodynia Testing (5 minutes): Allodynia is pain due to non-painful stimuli. Testing is done by using a Q-tip to touch 7 spots on the face and 7 spots inside the mouth and having each touch rated on a 0-100 pain scale.

4. Blood Draw (15 minutes): You will be escorted to the closest UWMC Laboratory Medicine Clinic for a blood draw. 2-3 teaspoons for blood will be drawn to test for levels of GRK2 which is related to pain sensitivity.

You may stop any test or refuse to answer question during the research visits.

RISKS, STRESS, OR DISCOMFORT

The risks in this study come from information from questionnaires, potential privacy loss, and adverse reactions to the pain sensitivity tests. The following provides a description of the possible risks associated with the different study procedures:

Questionnaires

The questionnaires may cause mild discomfort, anxiety, or stress. For example, you will be asked questions about how pain may affect your daily life. You can talk to the research team to discuss any discomfort, and you will be provided with contact information where you can seek care by a mental health professional or facility.

Pain sensitivity tests

There could be some discomfort when performing the pain sensitivity tests. You are asked only to complete these tests if you feel comfortable and safe. The pressure test may feel like someone is pressing down on your toe, your shoulder blade, or your cheek. The electrical stimulation may feel like a tingling sensation and could feel sore afterwards. The windup test may feel like someone is poking the top of your foot, shoulder blade, or your cheek.

Blood draw

Some people find blood draws uncomfortable. There is a risk of pain, bruise at the point where the blood is taken, redness or swelling of the vein and infection, and a rare risk of fainting.

Privacy

Although we will make every effort to keep your information confidential, no system for protecting your confidentiality can be completely secure. It is possible that persons might discover that you are in this study, or might obtain information about you.

ALTERNATIVES TO TAKING PART IN THIS STUDY

Being in this study is voluntary. You may refuse to participate and you are free to withdraw from the study at any time without penalty or loss of benefit to which you are otherwise entitled. Participating or not participating will not affect your clinical care in any way.

BENEFITS OF THE STUDY

Taking part in this research study will be of no direct benefit to you. However, knowledge may be gained that will benefit others in the future.

It is not the purpose of this research project to look for or provide you with any medical information or diagnoses.

CONFIDENTIALITY OF RESEARCH INFORMATION

Your participation in this study, and the information we gather from you will be kept confidential. The information we collect as part of this research study will not be included in your medical record. We will code your study information. We will keep the link between your name and your study information in a locked file at the University of Washington. Your study data will be kept indefinitely but will only be linked until December 31, 2019. Only the investigators listed above will have access to your identifiable data unless otherwise required by law. Although we will make every effort to keep your information confidential, no system for protecting your confidentiality can be completely secure. It is possible that unauthorized persons might discover that you are in this study, or might obtain information about you.

All of the information you provide will be confidential. However, if we learn that you intend to harm yourself or others, we must report that to the authorities.

Government or university staff sometimes review studies such as this one to make sure they are being done safely and legally. If a review of this study takes place, your records may be examined. The reviewers will protect your privacy. The study records will not be used to put you at legal risk of harm.

We will share what we learn with other health professionals through medical publications. None of these publications will include information that could identify you in any way.

OTHER INFORMATION

You may refuse to participate and you are free to withdraw from this study at any time without penalty or loss of benefits to which you are otherwise entitled.

You will receive $100 for completion of the study. If visit is split into two visits, you will receive $70 for the blood draw and $30 if all procedures except the blood draw is completed. You may be asked for your Social Security number for University of Washington record keeping.

RESEARCH-RELATED INJURY

If you think you have a medical problem or illness related to this research, contact Michele Curatolo at (206) 543-2568 right away. He will treat you or refer you for treatment.

Printed name of study staff obtaining consent Signature Date

Subject's statement

This study has been explained to me. I volunteer to take part in this research. I have had a chance to ask questions. If I have questions later about the research, or if I have been harmed by participating in this study, I can contact one of the researchers listed on the first page of this consent form. If I have questions about my rights as a research subject, I can call the Human Subjects Division at (206) 543-0098. I will receive a copy of this consent form.

Printed name of subject Signature of subject Date

Copies to: Researcher
Subject

B. PRESCREENING QUESTIONNAIRES

Method of Pre-Screening:
☐ EMR ☐ In Person ☐ Telephone ☐ Other:

Contact Info: Email: _____Telephone: _____

Initial Questions

- Subject Group: ☐ Control ☐ Fibromyalgia ☐ TMD
- English-speaking? Yes / No
- Female? Yes / No
- Age: _____
  - ≥ 18yo? Yes / No, Postmenopausal Yes/NO; if NO, expecting period_____
- Average daily pain score (0-10): _____
  - In FM and TMD candidates: Must be ≥ 3/10
  - In control candidates: No continuous pain lasting > 1 day during the last 3 months
    No pain at time of testing
- Daily/chronic pain duration: _____ (in years and/or months)
  - In FM and TMD candidates: Must be ≥ 3 months
- Opioid Use w/in previous 3 months? Yes / No
  - If Yes, list medication & dose info: _____
  - Opioid Pain Medication: codeine, fentanyl (Actiq, Duragesic, Fentora), hydrocodone (Hysingla ER, Zohydro ER), hydrocodone/acetaminophen (Lorcet, Lortab, Norco, Vicodin), hydromorphone (Dilaudid, Exalgo), meperidine (Demerol), methadone (Dolophine, Methadose), morphine (Astramorph, Avinza, Kadian, MS Contin, Ora-Morph SR), oxycodone (OxyContin, Oxcta, Roxicodone), oxycodone/Acetaminophen (Percocet, Endocet, Roxicet), oxycodone/naloxone (Targiniq ER)
- Currently pregnant? Yes / No
- Currently breastfeeding? Yes / No
- Currently using hormone replacement therapy within 30 days? Yes/No

- Currently using hormonal contraceptive within 30 days? Yes/No
- Current Endometriosis as diagnosed by a gynecologist of endometriosis? Yes/No
- Current infection? Yes / No **Any infx, other than minor cutaneous infxs at non-testing sites, are disqualifiers*
- Diagnosed immune or autoimmune disease? Yes / No
- Diagnosed neurologic disease? Yes / No
- Current migraine as diagnosed by a provider? Yes/No
- Diagnosed psychiatric disease, other than anxiety and/or depression? Yes / No

Is the candidate eligible to be a study subject? YES / NO

C. FM & TMD DIAGNOSIS FORMS

New Clinical Fibromyalgia Diagnostic Criteria – Part 1.

To answer the following questions, patients should take into consideration

- how you felt the **past week**,
- while taking your current therapies and treatments, and
- exclude your pain or symptoms from other known illnesses such as arthritis, Lupus, Sjogren's, etc.

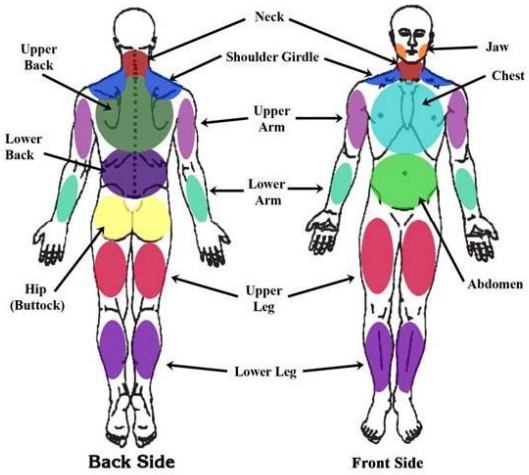
Subject ID: _____

Date: _____

Determining Your Widespread Pain Index (WPI)

Check each area you have felt pain in over the past week.

- ☐ Shoulder girdle, left
- ☐ Shoulder girdle, right
- ☐ Upper arm, left
- ☐ Upper arm, right
- ☐ Lower arm, left
- ☐ Lower arm, right
- ☐ Hip (buttock) left
- ☐ Hip (buttock) right
- ☐ Upper leg left
- ☐ Upper leg right
- ☐ Lower leg left
- ☐ Lower leg right
- ☐ Jaw left
- ☐ Jaw right
- ☐ Chest
- ☐ Abdomen
- ☐ Neck
- ☐ Upper back
- ☐ Lower back
- ☐ None of these areas



Count up the number of areas checked and enter your Widespread Pain Index or WPI score score here _____.

Symptom Severity Score (SS score) - Part 2a.

Indicate your level of symptom severity over the past week using the following scale.

Fatigue

- ☐ 0 = No problem
- ☐ 1 = Slight or mild problems; generally mild or intermittent
- ☐ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ☐ 3 = Severe: pervasive, continuous, life disturbing problems

Waking unrefreshed

- ☐ 0 = No problem
- ☐ 1 = Slight or mild problems; generally mild or intermittent
- ☐ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ☐ 3 = Severe: pervasive, continuous, life disturbing problems

Cognitive symptoms

- ☐ 0 = No problem
- ☐ 1 = Slight or mild problems; generally mild or intermittent
- ☐ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ☐ 3 = Severe: pervasive, continuous, life disturbing problems

Tally your score for Part 2a (not the number of checkmarks) and enter it here _____.

Symptom Severity Score (SS score)- Part 2b

Check each of the following OTHER SYMPTOMS that you have experienced over the <u>past week?</u>

- ☐ Muscle pain
- ☐ Irritable bowel syndrome
- ☐ Fatigue/tiredness
- ☐ Thinking or remembering problem
- ☐ Muscle Weakness
- ☐ Headache
- ☐ Pain/cramps in abdomen
- ☐ Numbness/tingling
- ☐ Dizziness
- ☐ Insomnia
- ☐ Depression
- ☐ Constipation
- ☐ Pain in upper abdomen
- ☐ Nausea
- ☐ Nervousness
- ☐ Chest pain
- ☐ Blurred vision
- ☐ Fever
- ☐ Diarrhea
- ☐ Dry mouth
- ☐ Itching
- ☐ Wheezing
- ☐ Raynauld's
- ☐ Hives/welts
- ☐ Ringing in ears
- ☐ Vomiting
- ☐ Heartburn
- ☐ Oral ulcers
- ☐ Loss/change in taste
- ☐ Seizures
- ☐ Dry eyes
- ☐ Shortness of breath
- ☐ Loss of appetite
- ☐ Rash
- ☐ Sun sensitivity
- ☐ Hearing difficulties
- ☐ Easy bruising
- ☐ Hair loss
- ☐ Frequent urination
- ☐ Painful urination
- ☐ Bladder spasms

Count up the number of symptoms checked above.
*If you tallied:

0 symptoms	Give yourself a score of 0
1 to 10	Give yourself a score of 1
11 to 24	Give yourself a score of 2
25 or more	Give yourself a score of 3

Enter your score for Part 2b here _____.

Now add Part 2a <u>AND</u> 2b scores, and enter ____.
This is your Symptom Severity Score (SS score), which can range from 0 to 12.

What Your Scores Mean

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 <u>AND</u> the SS score (Part 2a & b) is greater than or equal to 5

OR

1b. The WPI score (Part 1) is from 3 to 6 <u>AND</u> the SS score (Part 2a & b) is greater than or equal to 9.

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

3. You do not have a disorder that would otherwise explain the pain.

For example:
If your WPI (Part 1) was 9 and your SS score (Parts 2a & b) was 6, then you **<u>would meet</u>** the new FM diagnostic criteria.

If your WPI (Part 1) was 5 and your SS score (Parts 2a & b) was 7, then you **<u>would NOT</u>** meet the new FM diagnostic criteria.

*The new FM diagnostic criteria did not specify the number of "Other Symptoms" required to score the point rankings from 0 to 3. Therefore, we estimated the number of symptoms needed to meet the authors' descriptive categories of:

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

* Wolfe F, *et al*. <u>Arthritis Care Res</u> 62(5):600-610, 2010.

For information about Fibromyalgia Network, call our office Monday through Friday, 9:00 a.m. to 5:00 p.m. (PST) at (800) 853-2929 or visit us online at www.fmnetnews.com.

This survey is not meant to substitute for a diagnosis by a medical professional. Patients should not diagnose themselves. Patients should always consult their medical professional for advice and treatment. This survey is intended to give you insight into research on the diagnostic criteria and measurement of symptom severity for fibromyalgia.

DC/TMD Examination Form

Date filled out (mm-dd-yyyy) ___-___-____

Subject ID_____ Examiner_____

1a. Location of Pain: Last 30 days (Select all that apply)

RIGHT PAIN				LEFT PAIN			
O None	O Temporalis	O Other m muscles	O Non-mast structures	O None	O Temporalis	O Other m muscles	O Non-mast structures
	O Masseter	O TMJ			O Masseter	O TMJ	

1b. Location of Headache: Last 30 days (Select all that apply)

O None O Temporal O Other — O None O Temporal O Other

2. Incisal Relationships Reference tooth O US #8 O US #9 O Other

Horizontal Incisal Overjet O If negative ___ mm — Vertical Incisal Overlap O If negative ___ mm — Midline Deviation Right O Left O N/A O ___ mm

3. Opening Pattern (Supplemental; Select all that apply)

O Straight O Corrected deviation — Uncorrected Deviation: O Right O Left

4. Opening Movements

A. Pain Free Opening ___ mm

B. Maximum Unassisted Opening ___ mm

C. Maximum Assisted Opening ___ mm

D. Terminated? (N) (Y)

	RIGHT SIDE				LEFT SIDE			
		Pain	Familiar Pain	Familiar Headache		Pain	Familiar Pain	Familiar Headache
B. Maximum Unassisted Opening	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)
	Masseter	(N) (Y)	(N) (Y)		Masseter	(N) (Y)	(N) (Y)	
	TMJ	(N) (Y)	(N) (Y)		TMJ	(N) (Y)	(N) (Y)	
	Other M Musc	(N) (Y)	(N) (Y)		Other M Musc	(N) (Y)	(N) (Y)	
	Non-mast	(N) (Y)	(N) (Y)		Non-mast	(N) (Y)	(N) (Y)	
C. Maximum Assisted Opening	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)
	Masseter	(N) (Y)	(N) (Y)		Masseter	(N) (Y)	(N) (Y)	
	TMJ	(N) (Y)	(N) (Y)		TMJ	(N) (Y)	(N) (Y)	
	Other M Musc	(N) (Y)	(N) (Y)		Other M Musc	(N) (Y)	(N) (Y)	
	Non-mast	(N) (Y)	(N) (Y)		Non-mast	(N) (Y)	(N) (Y)	

5. Lateral and Protrusive Movements

A. Right Lateral ___ mm

B. Left Lateral ___ mm

C. Protrusion ___ mm

O If negative

	RIGHT SIDE				LEFT SIDE			
		Pain	Familiar Pain	Familiar Headache		Pain	Familiar Pain	Familiar Headache
A. Right Lateral	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)
	Masseter	(N) (Y)	(N) (Y)		Masseter	(N) (Y)	(N) (Y)	
	TMJ	(N) (Y)	(N) (Y)		TMJ	(N) (Y)	(N) (Y)	
	Other M Musc	(N) (Y)	(N) (Y)		Other M Musc	(N) (Y)	(N) (Y)	
	Non-mast	(N) (Y)	(N) (Y)		Non-mast	(N) (Y)	(N) (Y)	
B. Left Lateral	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)
	Masseter	(N) (Y)	(N) (Y)		Masseter	(N) (Y)	(N) (Y)	
	TMJ	(N) (Y)	(N) (Y)		TMJ	(N) (Y)	(N) (Y)	
	Other M Musc	(N) (Y)	(N) (Y)		Other M Musc	(N) (Y)	(N) (Y)	
	Non-mast	(N) (Y)	(N) (Y)		Non-mast	(N) (Y)	(N) (Y)	
C. Protrusion	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)	Temporalis	(N) (Y)	(N) (Y)	(N) (Y)
	Masseter	(N) (Y)	(N) (Y)		Masseter	(N) (Y)	(N) (Y)	
	TMJ	(N) (Y)	(N) (Y)		TMJ	(N) (Y)	(N) (Y)	
	Other M Musc	(N) (Y)	(N) (Y)		Other M Musc	(N) (Y)	(N) (Y)	
	Non-mast	(N) (Y)	(N) (Y)		Non-mast	(N) (Y)	(N) (Y)	

Diagnostic Criteria for Temporomandibular Disorders
Symptom Questionnaire

Subject ID_____ Date _____

PAIN

1. Have you ever had pain in your jaw, temple, in the ear, or in front of the ear on either side? — **No** ☐ **Yes** ☐

   If you answered NO, then skip to Question 5.

2. How many years or months ago did your pain in the jaw, temple, in the ear, or in front of the ear first begin? _____years _____months

3. In the last 30 days, which of the following best describes any pain in your jaw, temple, in the ear, or in front of the ear on either side?

   Select ONE response.

   ☐ No pain
   ☐ Pain comes and goes
   ☐ Pain is always present

   If you answered NO to Question 3, then skip to Question 5.

4. In the last 30 days, did the following activities change any pain (that is, make it better or make it worse) in your jaw, temple, in the ear, or in front of the ear on either side?

		No	Yes
A.	Chewing hard or tough food	☐	☐
B.	Opening your mouth, or moving your jaw forward or to the side	☐	☐
C.	Jaw habits such as holding teeth together, clenching/grinding teeth, or chewing gum	☐	☐
D.	Other jaw activities such as talking, kissing, or yawning	☐	☐

HEADACHE

5. In the last 30 days, have you had any headaches that included the temple areas of your head? — **No** ☐ **Yes** ☐

   If you answered NO to Question 5, then skip to Question 8.

6. How many years or months ago did your temple headache first begin? _____years _____months

7. In the last 30 days, did the following activities change any headache (that is, make it better or make it worse) in your temple area on either side?

		No	Yes
A.	Chewing hard or tough food	☐	☐
B.	Opening your mouth, or moving your jaw forward or to the side	☐	☐
C.	Jaw habits such as holding teeth together, clenching/grinding, or chewing gum	☐	☐
D.	Other jaw activities such as talking, kissing, or yawning	☐	☐

JAW JOINT NOISES				Office use		
8.	In the last 30 days, have you had any jaw joint noise(s) when you moved or used your jaw?	No ☐	Yes ☐	R ☐	L ☐	DNK ☐
CLOSED LOCKING OF THE JAW						
9.	Have you ever had your jaw lock or catch, even for a moment, so that it would not open ALL THE WAY? **If you answered NO to Question 9 then skip to Question 13.**	☐	☐	☐	☐	☐
10.	Was your jaw lock or catch severe enough to limit your jaw opening and interfere with your ability to eat?	☐	☐	☐	☐	☐
11.	In the last 30 days, did your jaw lock so you could not open ALL THE WAY, even for a moment, and then unlock so you could open ALL THE WAY? **If you answered NO to Question 11 then skip to Question 13.**	☐	☐	☐	☐	☐
12.	Is your jaw currently locked or limited so that your jaw will not open ALL THE WAY?	☐	☐	☐	☐	☐
OPEN LOCKING OF THE JAW						
13.	In the last 30 days, when you opened your mouth wide, did your jaw lock or catch even for a moment such that you could not close it from this wide open position? **If you answered NO to Question 13 then you are finished.**	☐	☐	☐	☐	☐
14.	In the last 30 days, when you jaw locked or caught wide open, did you have to do something to get it to close including resting, moving, pushing, or maneuvering it?	☐	☐	☐	☐	☐

D. JFLS-20, PHQ-9, GAD-7

Jaw Functional Limitation Scale – 20

For each of the items below, please indicate the level of limitation **during the last month**. If the activity has been completely avoided because it is too difficult, then circle '10'. If you avoid an activity for reasons other than pain or difficulty, leave the item blank.

		No limitation										**Severe limitation**
1.	Chew tough food	0	1	2	3	4	5	6	7	8	9	10
2.	Chew hard bread	0	1	2	3	4	5	6	7	8	9	10
3.	Chew chicken (e.g., prepared in oven)	0	1	2	3	4	5	6	7	8	9	10
4.	Chew crackers	0	1	2	3	4	5	6	7	8	9	10
5.	Chew soft food (e.g., macaroni, canned or soft fruits, cooked vegetables, fish)	0	1	2	3	4	5	6	7	8	9	10
6.	Eat soft food requiring no chewing (e.g., mashed potatoes, apple sauce, pudding, pureed food)	0	1	2	3	4	5	6	7	8	9	10
7.	Open wide enough to bite from a whole apple	0	1	2	3	4	5	6	7	8	9	10
8.	Open wide enough to bite into a sandwich	0	1	2	3	4	5	6	7	8	9	10
9.	Open wide enough to talk	0	1	2	3	4	5	6	7	8	9	10
10.	Open wide enough to drink from a cup	0	1	2	3	4	5	6	7	8	9	10
11.	Swallow	0	1	2	3	4	5	6	7	8	9	10
12.	Yawn	0	1	2	3	4	5	6	7	8	9	10
13.	Talk	0	1	2	3	4	5	6	7	8	9	10
14.	Sing	0	1	2	3	4	5	6	7	8	9	10
15.	Putting on a happy face	0	1	2	3	4	5	6	7	8	9	10
16.	Putting on an angry face	0	1	2	3	4	5	6	7	8	9	10
17.	Frown	0	1	2	3	4	5	6	7	8	9	10
18.	Kiss	0	1	2	3	4	5	6	7	8	9	10
19.	Smile	0	1	2	3	4	5	6	7	8	9	10
20.	Laugh	0	1	2	3	4	5	6	7	8	9	10

Subject ID _____

Patient Health Questionnaire - 9

Date _____

Over the last 2 weeks, how often have you been bothered by the following problems? Please place a check mark in the box to indicate your answer.

	Not at all	Several days	More than half the days	Nearly every day
	0	1	2	3
1. Little interest or pleasure in doing things	☐	☐	☐	☐
2. Feeling down, depressed, or hopeless	☐	☐	☐	☐
3. Trouble falling or staying asleep, or sleeping too much	☐	☐	☐	☐
4. Feeling tired or having little energy	☐	☐	☐	☐
5. Poor appetite or overeating	☐	☐	☐	☐
6. Feeling bad about yourself – or that you are a failure or have let yourself or your family down	☐	☐	☐	☐
7. Trouble concentrating on things, such as reading the newspaper or watching television	☐	☐	☐	☐
8. Moving or speaking so slowly that other people could have noticed? Or the opposite – being so fidgety or restless that you have been moving around a lot more than usual	☐	☐	☐	☐
9. Thinking that you would be better off dead or of hurting yourself in some way	☐	☐	☐	☐
TOTAL SCORE =				

If you checked off any problems, how difficult have these problems made it for you to do your work, take care of things at home, or get along with other people?

Not difficult at all	Somewhat difficult	Very Difficult	Extremely difficult
☐	☐	☐	☐

GAD - 7 Subject ID _____

Date _____

Over the last 2 weeks, how often have you been bothered by the following problems? Place a check mark in the box to indicate your answer.

	Not at all	Several days	More than half the days	Nearly every day
	0	1	2	3
1. Feeling nervous, anxious or on edge	☐	☐	☐	☐
2. Not being able to stop or control worrying	☐	☐	☐	☐
3. Worrying too much about different things	☐	☐	☐	☐
4. Trouble relaxing	☐	☐	☐	☐
5. Being so restless that it is hard to sit still	☐	☐	☐	☐
6. Becoming easily annoyed or irritable	☐	☐	☐	☐
7. Feeling afraid as if something awful might happen	☐	☐	☐	☐
TOTAL SCORE =				

If you checked off any problems, how difficult have these problems made it for you to do your work, take care of things at home, or get along with other people?

Not difficult at all	Somewhat difficult	Very difficult	Extremely difficult
☐	☐	☐	☐

E. DEMOGRAPHIC & GENERAL PAIN ASSESSMENT FORM

GPCRK2 AS A REGULATOR OF CENTRAL SENSITIZATION IN FIBROMYALGIA AND CHRONIC TEMPOROMANDIBULAR DISORDER

SUBJECT ID _____

DATE _____

DEMOGRAPHIC & GENERAL PAIN ASSESSMENT FORM

Please fill out the following health history information:

Date of Birth: _____ Height: _____

Age: _____ Weight: _____

Ethnicity & Race:
(Select all that apply)

- ☐ African American/Black
- ☐ American Indian/Native Alaskan
- ☐ Asian/Pacific Islander
- ☐ Hispanic/Latino
- ☐ Caucasian

Please provide a list of your medications with dosage and usage:
(Include ALL prescription, including hormonal contraceptives, over-the-counter and homeopathic/alternative forms)

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

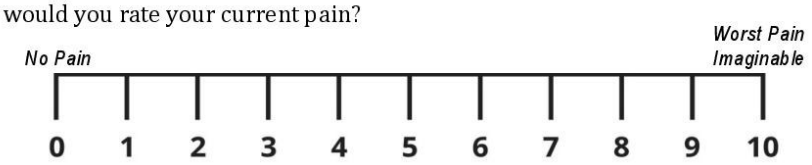
GPCRK2 AS A REGULATOR OF CENTRAL SENSITIZATION IN FIBROMYALGIA AND CHRONIC TEMPOROMANDIBULAR DISORDER

SUBJECT ID _____

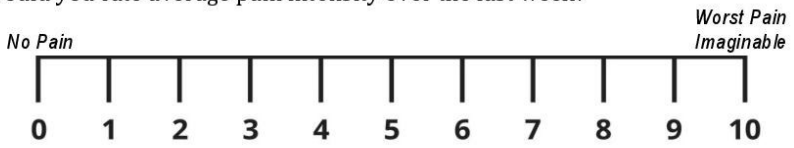
DATE _____

Using the numeric scales below, with 0 representing no pain and 10 representing the worst pain imaginable, please rate the following two questions:

- How would you rate your current pain?

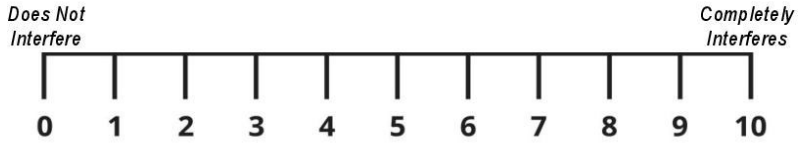


- How would you rate average pain intensity over the last week?

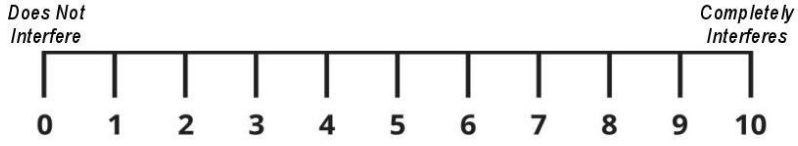


Using the numeric scales below, with 0 representing no interference and 10 representing complete interference, please rate your pain's interference with the following:

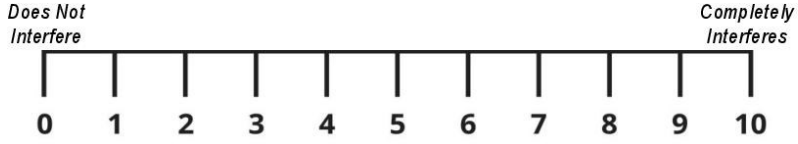
- General activity



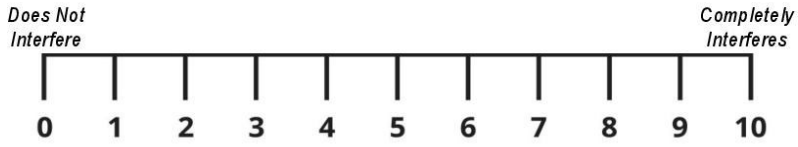
- Enjoyment of life



- Falling asleep



- Staying asleep



F. SCREENING AND TESTING RECORD

GRK2 AS A REGULATOR OF CENTRAL SENSITIZATION IN FIBROMYALGIA AND CHRONIC TEMPOROMANDIBULAR DISORDER
#52228

SUBJECT ID _____
⊡CONTROL ⊡TMD ⊡ FM
DATE _____

Screening & Testing Record

Assessment Form

☐ BMI:
Weight: _____lb ----------> _____kg
Height: _____in --> _____cm --> _____m --> _____m^2

Screening

☐ Menstrual Cycle: Cycle Length = _____days (< 21 or > 35 excluded)
Day of Cycle = _____ (on day of testing)

☐ Navigate Pain App:
Pixel Area: _____

☐ PHQ-9: _____
Function Difficulty: ☐None ☐Somewhat ☐Very ☐Extremely

☐ GAD-7: _____
Function Difficulty: ☐None ☐Somewhat ☐Very ☐Extremely

☐ FM Criteria: WPI #1 _____ (0-19) + SS 2a _____ (0-9) + SS 2b (0-3) = _____

☐ JFLS–20: _____ (0-80)

☐ DC-TMD Symptom Questionnaire

☐ DC-TMD Examination

Testing

Blood Sample:

☐ Collected; date _____time_____
☐ Transferred to Eoin's lab; date _____
☐ Results received; date _____

GRK2 AS A REGULATOR OF CENTRAL SENSITIZATION IN FIBROMYALGIA AND CHRONIC TEMPOROMANDIBULAR DISORDER
#52228

SUBJECT ID _____
⊡CONTROL ⊡TMD ⊡ FM
DATE _____

☐ Dynamic mechanical allodynia testing (0-100)

Nerve	Extraoral sites-skin of face Nerve branch-test location	Q-tip Right	Left
V1-SO	Supraorbital nerve: forehead in line with pupil		
V2-IO	Infraorbital nerve: lateral to ala of nose in line with pupil		
V3 MN	Mental nerve - chin1/2 way -lip commissure to midline		
V3-AT	Auriculo-Temporal nerve: 5 mm. anterior to tragus		
V3 LB	Long buccal nerve cheek to 1 cm posterior to lip commissure		
C2/3	On superior Sternocleidomastoid		

Nerve	Intraoral sites-on gingiva/tongue Nerve branch-test location	Q-tip Right	Left
V2-ASA	Anterior Sup alveolar n.- buccal #7/#10		
V2-PSA	Post. Sup Alveolar n.-buccal #3/#10		
V2-NP	Nasopalatine n.- palatal #7/#10		
V2-GP	Greater Palatine n.- palatal to #3/#14		
V3-M	Intraoral Mental n.-gingiva#22/#27		
V3-ILB	Intraoral long buccal n.-gingiva#19/#30		
V3-LiG	Lingual n. – lingual gingiva #21/#28		
V3-LiT	Lingual n. – mid dorsum tongue		

"Do you feel it more on the right, more on the left, or does it feel the same?
Was this stimulus painful on the right, on the left, both sides or neither?"

☐ Pressure Algometry (1-1000kPa)

Training session (Palm)	Periscapular	Masseter	2nd Toe
Actual PPT			

G. BLOOD PROTOCOL

This section is adopted verbatim from pilot study-A (106).

G1. Whole blood stimulation protocol

1) Warm stimulation plate and 14 mL RPMI aliquot for 10 minutes at 37°C. Wipe condensation with paper towel.
2) Rotate citrated blood tubes 10x, place in first row of white rack with sticker facing away, and discard blue cap.
3) Peel Aluminum seal off stimulation plate. Tilt plate to confirm all wells in columns 1-5 have RPMI + stimuli.
4) Use sterile razor blade to open reservoir and any remaining Aluminum seal on stuck stim plate when removed.
5) Use 10 mL stripette to aspirate blood from both tubes and dispense into 50 mL tube.
6) Measure the volume of citrated whole blood and add an equal volume of warmed RPMI.
7) Place cap on tube containing blood and rotate in hand 10x and pour into reservoir. There should be no bubbles.
8) Place 10 mL stripette into 50 mL tube that blood was poured out of to drain any blood in stripette.
9) Evenly press tips onto manual 8 channel P1000 set to 380 µL.
    a) Examine tips to make sure there is no gap between filter and top of pipette shaft.

b) Pre-wet tips in blood one time without pushing past first stop on pipette.

c) Check for equal volumes in all tips.

d) Evenly insert all tips to bottom left side of wells in column 1 of stimulation plate. To ensure tips are on the bottom side of well, and not at the dimpled bottom of well, tilt pipette 10 degrees to the right when inserting.

e) Mix 3x without creating bubbles i.e.do not push past first stop on pipette after each mix. Place reservoir next to stim plate to hold plate in place while mixing.

f) After 3rd mix pull tips up the side of the well with slight tilt to the right, pause for 3 seconds, and push past pipette stop at top of blood level by visual inspection (about 2/3rds up the side of the well).

If push past first pipette stop at very top of well some blood will remain at top of well and not mix.

g) Un-tilt tips until vertical, then bring to the center of the wells and pull up without touching sides of wells.

10) Eject pipette tips in discard box and repeat step 9 to fill remaining 4 columns with blood.

11) Seal plate with Breathe-Easy Seal:

a) Hold seal so that the clear plastic portion, which is sticking out past white tab, is in left hand.

b) Pull white backing off the bottom of the seal.

c) Holding the white tabs at both ends, bow the sticky bottom by bringing hands closer together.

d) Place bowed bottom in the middle of plate and push in even motion to the ends of plate.

e) Press slick side of removed bottom white backing on top of next layer to form a tight seal of all wells.

f) Push white tabs overhanging ends of plate down vertical on both outside ends of the plate.

If the white tab on column 12 is on the top of the plate this is acceptable.

The white tab on the left needs to overhang column 1 end of plate.

g) Holding the white tab vertical on the left peel off clear top plastic sheet.

h) Use sterile side of plastic sheet just pulled off to press down seal to assure there a depression in each well.

i) Confirm all wells are sealed and if necessary repeat step h).

j) Place blood reservoir tilted on front metal lip of hood to allow blood to drain to end facing away from tilt.

12) Place plate on Lab-Line Titer shaker for 15 minutes on Constant setting of 6, RM 620D.

Note: This 15 minute incubation happens while preparing the PBMCs for PBS washing so keep an eye on the 15 minute timer. Plan accordingly to make sure the plate does not shake longer than 15 minutes.

13) Record incubation start time in notebook and later in Excel PAIN log sheet. Incubation start time is recorded as the time the plate was first put on shaker.

14) Place plate in bottom of EW 37°C 5% CO_2 incubator for 24 hours RM 615C. Check water level in metal tray at bottom of incubator. If necessary add autoclaved water.

15) Transfer any blood in reservoir, 50 mL tube and citrated tubes into a labeled 15-mL conical tube.

   a) Place blood on ice until PBMC lysate is stored in MW freezer #2 Cat Woman -80°C bottom shelf second rack from right.

   b) Place blood tube upright in far-right bottom rack wedged upright in paper towel on front of rack until frozen then place in PAIN Blood box. Placement into box can wait until the following day.

      Discard glass tubes into sharpie container, not pipette tip discard box.

G2. Plasma harvest protocol

Reagents and Supplies:

- Rainin EDP-3 P-200 electronic pipette
- Polypropylene 96 well plate Thermo Scientific Prod#: 267334 U96 PP 0.5 mL (need 3)
- Aluminum Sealing Tape Corning REF 6570 (3)
- Freezer labels appropriate for -80□C
- Razor blade

1) After 24 hours, pre-chill Sorvall RT6000 centrifuge (SA Lukehart) located outside RM 620D to 4°C.
2) Review electronic pipette tips below if not familiar with electronic pipette.
3) Label 3 freezer stickers with patient ID Sup 1, 2 or 3 and apply to end (column 1 A-H) of 96-well polypropylene plates.
    a) After firmly applying labels in hood use razor blade to cut off bottom part of excess sticker.
    b) Use Fisher Pen to label front and side of plates with patient ID, Sup # and date.

    The freezer labels are unused sections of sheets provided by MW ACCESS study.

    The Labels are collected in tray next to barcode reader in room 610 or behind my desk in room 626.
4) Use pre-aliquoted balance plate and centrifuge plates for 10 minutes at 1200 rpm, 300 g 4°C.
5) Holding both ends of Breath-Easy Seal covering column 1 peel off ½ way off plate and evenly place seal over opposite end of plate without creating wrinkles in seal.
6) Use P200 electronic pipette, speed 4, Multi-Mode 3x, set to 66.66 µL to aspirate 200 µL of plasma supernatant from first column of stimulation plate. Dispense 66.66 µL into the same column of 3 separate 96-well polypropylene plates labeled with patient ID, Sup 1,

2, 3 and date. Insert pipette tips into a column of wells until feel the second ridge of pipette tips catch top lip of column.

7) Change pipette Mode to Single, aspirate 50 uL of plasma and dispense into Supernatant plate #3.
8) Repeat this procedure on the next 4 columns of stimulation plate.
9) Pull back Breath-Easy seal to cover any remaining blood in the stimulation plate and discard in autoclave bag.
10) If at any time whole blood was aspirated eject all volume into correct wells, re-seal, re-spin and re-aliquot.
11) Seal each plate with aluminum seal. Use slippery side of seal backing to press hard enough that the letters in row A-H should be visible thru the seal and all wells are dimpled.
12) Use razor blade to cut off any excess foil over the front side of the plate making especially sure to cut off the aluminum tab. Also press Aluminum seal down the notched corner of the plate so this tab is not sticking out.

    If tab catches on anything while at -80°C the entire seal will lift off.
13) Place Supernatant harvest plates in -80°C Vostok where the original stimulation plate was taken from.

    Electronic pipette tips: Check settings below prior to using pipette. Changes can be made as follows:

    a) Touch the MODE button on Rainin 8 channel multi-channel pipette to advance into MULTI mode (should only take one click).

b) Use arrows to scroll to 66.66 µL.

c) Touch RESET, set to 3x with up or down arrow and when get to 3x touch RESET and hit enter.

d) Make sure pipette shows PICKUP, now you are ready to aspirate the 200 uL and dispense 66.66 uL into 3 plates.

e) Dispense plasma with tips on the bottom well of plate and pull up the side of the plate when finishing dispense. Pull tips out of center of well prior to getting to top of well.

f) When dispensing the 3rd plate hit the RESET button to dispense all the supernatant in 3rd plate and pull tips out as soon as fluid is dispensed or it will create bubbles and aspirate volume.

g) Use Rainin P200 filter tips.

h) Press tips on evenly without any tilt (gaps at top of where the tips go up channel shaft).

i) Do not rock pipette back and forth to get tips on or one end will be uneven.

j) Always make sure the pipette is in the PICKUP mode prior to aspirating supernatants.

k) Place the tips into the column wanting to harvest at a slight angle 10-20 degrees from vertical and you will feel the first ridge on outside of the tips, when get to SECOND ridge hit aspirate.

l) If you cannot aspirate the full 200 uL check to see that pipette speed is 3 using the MODE button.

m) If you still cannot aspirate the full 200 uL after reaching 2nd ridge bring tips to nearly vertical, touch the aspirate button to pull up 200 uL or the 50 µL for supernatant plate 3.

To Aspirate while tips are vertical:

i) Insert pipette tips into a column of wells until feel the second ridge of pipette tips catch top lip of column

ii) Use non-pipette hand index finger to support tip in row 1 to ensure tips don't pass 2nd ridge of tip.

iii) Press pipette slightly to the right with no downward force.

iv) Rock pipette to top and back of plate 5-10 degrees until feel all tips resting on second tip ridge.

v) Keep using index finger to support pipette and bring tips to nearly vertical then touch the aspirate button.

Make sure pipette is in the PICKUP mode prior to placing on charger otherwise it will not charge

G.3 Whole blood lysate protocol

Whole blood cell lysate protocol in each well after 24 hour culture

Reagents and Supplies:

- P1200, 200, 20 pipettes and filter tips
- Stimulation plate cultured for 24 hours
- Aluminum Sealing Tape Corning REF 6570 that originally was stuck on stim plate and stuck under hood
- RBC Lysis Buffer, 10X Santa Cruz sc-296258

- Timer

1) Add 5 mL of 10x RBC lysis buffer to 45 mL deionized water for 1x working dilution. Keep at room temp.
2) Pour 25 mL 1x RBC lysis buffer into reservoir rinsed and saved for RBC lysis.
3) Add 300 µL 1x RBC lysis buffer with same set of tips for all wells. Note time started adding to column 1.
4) Use P200 filtered tip with 8-channel manual pipette set to 100 uL mix each column 10 x or more until RBC clumps are dissolved.
5) Use Breathe-Easy end that was not covering the blood during stimulation or use a new plastic seal to cover.
6) Place plate on Lab-line shaker setting 10 Constant for 30 seconds.
7) After 5 minutes in RBC lysis buffer centrifuge 5 min at 1600 rpm 4°C.
8) Use one sterile 9-inch glass pipette to aspirate the supernatant from each well.

Use left hand to tilt the plate about 45 degrees with blood in A1-H5 closest to front of hood.

Insert glass pipette to well A1 until reaches bottom lip of well closest (6 o'clock position) in 1 second. The glass pipette should have slight pressure on side of well, pipette may even bend a little. Proceed to well B1 etc. until all wells have been aspirated 1 second from start of aspiration to bottom of well

9) Go back to each well and aspirate any fluid droplets that are on top of bottom well lip above pellet, repeat.
10) Add 400 µL of 1x RBC lysis buffer, and seal.
11) If RBC clumps still are present use unfiltered P200 tips to mix 10x until RBC coagulated RBCs are gone.
12) Place on shaker for 30 seconds, setting of 10 C.
13) After 5 minutes in 2nd round RBC lysis buffer centrifuge 5 min at 1600 rpm 4°C.
14) Use one sterile 9-inch glass pipette to aspirate the supernatant from each well and repeat aspiration.
15) Add 400 µL PBS to washed and saved PBS reservoir and centrifuge 5 min at 1600 rpm 4°C.Time permitting take a 50 uL aliquot from wells C5 and D5 for cell count and cytospin differential.
16) Aspirate PBS, place plate on ice.
17) Add 25µL of cold RIPA+PI to each well using the same tip inserted in center near bottom of well.
18) Cover with aluminum seal saved from stimulation plate stuck under fume hood the day before.

    Place on ice 15 minutes, vortex 1 minutes, and place plate at -80C VoStok.

G4. Isolation of PBMCs using cell processing tubes (CPT)

<u>PBMC Isolation Supplies:</u>

- 15 mL tubes

- 3 mL Falcon Transfer Pipet Sterile Cat. No. 357575
- Phosphate Buffered Saline without Calcium and Magnesium
- Turk's Solution EMD Millipore Cat. No. 109277
- RIPA Buffer Sigma-Aldrich Cat. No. R 0278
- Protease Inhibitor Cocktail Set III, Calbiochem çat. No. 539134 Lot: 2888287
- Bio-Freeze vials with gasket- Sterile Screw Tube w/Standard Cap Color Natural 0.5mL, GeneMate Cat. No C-3273-1
- Wash buffer PBS containing 2 mM EDTA and 0.5% very low endotoxin BSA

Blood Draw Supplies:

- BD Vacutainer Safety-Lok 21G REF 367281
- BD Vacutainer One-Use Non-Stackable Holder
- BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate REF 362761 8 mL Draw Capacity *We Provide CPT* https://www.bdj.co.jp/pas/products/mekkin/1f3pro00000r5drz-att/bd-cpt-manual-362760-362761.pdf

Protocol: Isolation of PBMC at R&T

1. Centrifuge CPT at 2800 rpm (1800g) 20°C for 20 minutes in Sorvall Legend XTR with NO BRAKE (EW-lab)
2. Aspirate upper cell free plasma layer with sterile 9" glass pipette
3. Transfer PBMC to 15 mL tube with a sterile plastic transfer pipet

4. Fill tube with ice cold RPMI+P/S to 15 mL, mix and take 25 uL for cell count while cells are pelleting in next step

    25 µL cells + 225 µL Turk's Solution: Dilution_____ x Count_____ x 2500 =_____cells/mL

5. Centrifuge at 400xg (1400 rpm) for 10 min at 4°C and aspirate supernatant
6. Resuspend cells in 1 mL ice cold wash buffer, transfer to 1.7 mL microfuge tube and spin briefly until cells are pelleted
7. Aspirate supernatant and repeat above step 6 for a second wash in ice cold WB
8. Resuspend pellet in 100 µL if ice cold RIPA buffer + protease inhibitors added just prior to this step
9. Incubate on ice for 30 min, flicking the tube every 10 min
10. Clarify the lysate by centrifugation at 13000 x g for 15 minutes at 4°C.
11. Carefully transfer the supernatant into one 0.5 ml a bio-freeze vial and then transfer ½ of supernatant to another tube
12. Place tubes in -80C
13. Log sample location and make sure this patient worksheet is filled out and sample location recorded

H. SUBJECT ENROLLMENT

This section is adopted verbatim from pilot study-A (106).

H1. Selection of Case Subjects

TMD: Patients from the Oral Medicine Clinical Service with a clinical diagnosis of temporomandibular disorders were invited by recruiters to participate. The clinic has multiple oral medicine specialists who manage TMD patients in a regular basis. TMD patients were also recruited via posted flyers, flyers given to subjects during clinic visits, and by letters sent out to eligible participants identified through the Medical Informatics Cohort Patient Identification, ITHS (Institute of Translational Health Sciences) service.

Potential TMD subjects who came to Oral Medicine Clinical Services between December were screened using a questionnaire (see attached questionnaire in the Appendices).

Fibromyalgia: We recruited fibromyalgia patients through several methods including flyers posted at the University of Washington Medical Center, letters sent out to participants of previous fibromyalgia studies, the ITHS (Institute of Translational Health Sciences) service, and inviting some patients with diagnosis of fibromyalgia from the Oral Medicine Clinic. Potential FM subjects were assessed for eligibility using the same pre-screening questionnaire as the TMD group.

Inclusion criteria:

1. Female
2. Age ≥ 18 years old
3. English speaker

4. Average daily pain at least 3 (0 = no pain, 10 = unbearable pain) in the past week.
5. Pain duration: ≥3 months
6. Only potential fibromyalgia participants who met the criteria of the American College of Rheumatology were enrolled in the study.(2) (Questionnaires & criteria in Appendices).
7. Only potential TMD participants who met the diagnosis of DC TMD (31) were enrolled (see Appendices).

Exclusion criteria

1. Pain score less than 3 at the time of testing
2. Opioid usage in previous 3 months
3. Pregnancy
4. Breastfeeding
5. Intake of hormonal contraceptives or systemic hormone replacement therapy in previous 30 days
6. Endometriosis as diagnosed by a gynecologist
7. Infection within one week prior to the testing day
8. Autoimmune diseases
9. Migraine diagnosed by a medical provider
10. Psychiatric disease, other than anxiety and depression.

H2. Selection of Control Subjects

Twenty four potential control subjects were screened using a pre-screening questionnaire (Appendices). Some control subjects were staff or students at the School of Dentistry, University

of Washington, or friends of tested subjects. Recruitment was also performed via posters, flyers, and letters sent to potential participants identified through the ITHS service.

Inclusion Criteria

1. Female
2. Age ≥ 18 years old
3. English speaker
4. No pain in any body site.
5. No diagnosis of tension-type headache based on the International Classification of Headache (ICHD-3)(129)

Exclusion criteria*

1. Any pain at the time of experimental session
2. Any headache on the testing day.

*Other exclusion criteria are the same as TMD and fibromyalgia participants, except for pain score.

I. STUDY SCHEDULE

This section is adopted verbatim from pilot study-A (106).

On the testing day, a screening questionnaire was administered to ensure participants' eligibility. They also gave written informed consent to participate after a detailed explanation of the study. In order to characterize the patient population, the following parameters were collected through supplemental questionnaires (see Appendices)

- Gender (subjects were all female)

- Age
- Body-mass index
- Ethnicity
- Duration of chronic pain (years since daily pain began)
- Current medications
- Pain intensity at the time of testing (assessed by a 0-10 numerical rating score (NRS), whereby 0 = no pain and 10 = unbearable pain)
- Average pain intensity during the last week, as assessed by the NRS
- Interference of pain with general activity (0-10)
- Interference of pain with enjoyment of life (0-10)
- Interference of pain with falling asleep (0-10)
- Interference of pain with staying asleep (0-10)
- Patient health questionnaire - 9 (PHQ-9) for the assessment of depression (130).
- Generalized anxiety disorder – 7 (GAD-7) for the assessment of anxiety (131).
- Fibromyalgia survey score for the quantification of fibromyalgia symptoms (2).
- The diagnostic criteria for temporomandibular disorders – Symptom questionnaire (31).
- Jaw functional limitation scale - 20 (JFLS-20) (132).

Fibromyalgia or TMD subjects who did not meet the diagnostic criteria on the day of testing were excluded from the study even though they had met diagnostic criteria previously.

TMD examination was done to confirm diagnosis in combination with history, following DC/TMD diagnostic criteria.(31)

After completion of questionnaires, 6 ml of blood was collected into each of two citrate tubes at the Research Testing Service (RTS), University of Washington Medical Center, and sent for standard hematologic profiles. Four ml. of blood was collected into one CPT mononuclear preparation tube containing sodium heparin. Samples were transported to Harborview Research and Training Building to process for IL1β level and GRK2 level on the same day. (See section 3.4.2 for details)

After blood draw, participants were tested in the Oral Medicine Clinical Service to assess for central sensitization indices. These measurements were collected in a consistent order on each subject. (See 3.4.1 for details)

J. METHODS

This section is adopted verbatim from pilot study-A (106).

J1. Assessment of Central Sensitization

J1.1 Pain area. This parameter was recorded before starting the assessment of pain and reflex thresholds. Patients drew their pain localization on a high resolution 3D body schema on a personal computer tablet (Samsung Galaxy note 10.1) using the Navigate pain app (Aalborg University, Denmark)

J1.2. Windup ratio. Windup ratio is a test of repeated mechanical stimulation with a standardized metal rod ranging from 8 to 512 mN. A

single stimulus was placed on the skin surface and the subject rated the pain on a scale of 0 to 100; then, after 10 seconds, single stimuli were placed every second, one per second, for a total of 10 times. The subject rated the pain after the first, and then after 10^{th} stimulus in a row. For FM patients, the stimulation was applied on the dorsum of the foot (5 cm caudal to the ankle joint), to the most painful spot of the periscapular region of the back and the middle of the masseter muscle. For TMD patients, the stimulation was performed on the dorsum of the foot (5 cm caudal to the ankle joint), the most painful spot of the masseter muscle (if there is any masseter tenderness, otherwise at the middle of the masseter muscle), and 2 cm cranial to the middle of scapular spine. For the control group, the sites were the dorsum of the foot (5 cm caudal to the ankle joint), 2 cm cranial to the middle of the scapular spine, and the middle of the masseter muscle.

J1.3. Pressure stimulation. Pain detection threshold was measured with an electronic pressure algometer. The pressure was applied at the following sites: 1) in the FM group, at the center of the pulp of the 2^{nd} toe, the most painful spot of the periscapular region, and the middle of the masseter; 2) in the TMD group, at the center of the pulp of the 2^{nd} toe, at the most painful spot of the masseter (if there was any masseter tenderness, otherwise at the middle of the masseter muscle), and 2 cm cranial to the middle of the scapular spine; 3) in the control group, at the center of the pulp of the 2^{nd} toe, 2 cm cranial to the middle of the scapular spine, and the middle of the masseter muscle. The probe has a surface area of 1 cm^2. The pressure was

increased from 0 to a maximum of 1000 kPa, at a rate of 30 kPa/s. Pain detection threshold was defined as the value at which the pressure sensation turned to pain.

J1.4. Electrical stimulation. Electrical stimulation was performed through surface electrodes placed at the arch of the foot (innervation area of the median plantar nerve). A train-of-five 1 ms square-wave pulses delivered at 200 Hz (perceived as a single stimulus) was used. Electromyography reflex responses to the stimulation were recorded from the anterior tibialis muscle by surface electrodes. The current intensity was increased from 1 mA in steps of 1 mA until 1) a reflex with an amplitude > 20 □V for at least 10 ms in the 70-150 ms post-stimulation interval was detected (nociceptive reflex threshold); and 2) a pain sensation was evoked (electrical pain threshold).

J2. Measurement of GRK 2 and TLRs

The blood sample was taken between 7.30 am and 5.45 pm. The average time between blood draw and blood stimulation was 2.5 hours. Blood processing and analyses were done at UW Medicine Research Facility. As the blood stimulation was conducted on a 96-well plate, other TLR agonists were also used to stimulate blood samples for future analysis. A brief blood protocol is summarized here.

Blood was collected into two 4.5 mL sodium citrate tubes, transported to the laboratory, pooled into one 15 mL tube, diluted 1:1 with warmed media. Then 380 µL was added to a 96-well plate containing 20 µL of 20x concentrated triplicates of innate

immunity stimulating agonists; Toll-like receptor (TLR)1/2 agonist (Pam3CYSK4) at 10 ng/ml, 100 ng/ml, 1000 ng/ml, TLR 4 agonist (lipopolysaccharide 011:B4) at 0.01, 0.1,1,10 ng/ml, TLR 7/8 agonist (derivative of the imidazoquinoline compound R848) at 250, 1000ng/ml. Monosodium urate (MSU) crystals at 1 and 10 μg/mL were also co-cultured with lipopolysaccharide to evaluate the effect of innate immunity (0.01 ng/mL, 0.1 ng/ml, 1 ng/ml).. Full details of the blood stimulation protocol are attached in appendix 7.7. Plates were incubated at 37 ºC, 5% CO2 for 24hr, centrifuged at 300 g for 10 minutes and supernatants from all wells were harvested (see details in Appendices). Samples were analyzed for IL1β using DuoSet ELISA Development kit (R&D system) which contains the basic components required for the development of sandwich ELISAs to measure natural and recombinant human IL1β/IL-1F2.