G protein-coupled Receptor Kinase 2 (GRK2) and Toll-like Receptors as Regulators of Central Sensitization in Fibromyalgia and Chronic Temporomandibular Disorders

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Abstract

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Background. Fibromyalgia (FM) and chronic temporomandibular disorders (TMD) are highly debilitating disorders characterized by pain that is not explained by tissue lesions. Central sensitization (CS) is proposed to be a common etiology of both syndromes. However, the mechanism underlying CS is unclear. Glial cell activation via toll-like receptor (TLR) signaling has been consistently demonstrated in animal models to play an essential role in the initiation and persistence of chronic pain. TLR activation also suppresses GRK2 expression, partly via the release of IL-1β. GRK2 expression and TLR activation are not measurable in human glial cells, but can be assessed in peripheral blood mononuclear cells (PBMCs). Preliminary studies have shown that that GRK2 expression and TLR activity are altered in PBMCs of chronic pain

patients, potentially providing a clinically accessible marker of a pathway involved in the pathophysiology of central sensitization.

Hypotheses. 1) In comparison to normal controls, patients with FM and chronic TMD display lower basal GRK2 levels in PMBCs. 2) Basal GRK2 levels are correlated negatively with indices of central sensitization.3) In comparison to normal pain-free controls, patients with FM and TMD display higher IL-1β production after TLRs stimulation. 4) IL1β levels are correlated negatively with all indices of CS.

Methods**.** We assessed IL1β and GRK2 level, as well as CS in 18 women with fibromyalgia, 18 with chronic TMD, and 18 pain-free female controls. Whole blood was stimulated in vitro by TLR 2, 4, 7 agonists at various concentrations. IL1β levels following stimulation were measured using ELISA. GRK 2 levels on PBMCs were visualized with fluorescent secondary antibodies and quantified using Western blot technique. CS was quantified by pain reports in response to pressure, electrical and repeated mechanical stimulation, areas of pain, and withdrawal reflex of the leg.

Results. Basal GRK 2 levels are pending to be analyzed by Western blot. The study, therefore, focused mainly on TLR 2 responsiveness as the preliminary data, No statistically significant difference was identified between the IL1β expression following TLR 2 stimulation among pain-free controls, TMD and FM subjects. Nociceptive reflex threshold was negatively correlated with IL1β level following TLR 2 (1000ng/ml) stimulation.

Conclusion. IL1β expression after TLR 2 stimulation in peripheral blood cells did not differ in FM participants and chronic TMD participants when compared to controls. The negative correlation between IL1β expression and spinal nociceptive reflex suggested that TLR 2

responsiveness via IL1β expression may be one of the mechanisms underlying central sensitization in the spinal cord.

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Chapter 1. INTRODUCTION

1.1 CENTRAL SENSITIZATION AND CHRONIC PAIN DISORDERS.

Fibromyalgia (FM) and chronic temporomandibular disorders (TMD) are very challenging chronic pain disorders that share some features. FM has a prevalence of 10-15% in the general population [1] and is associated with high disability levels [2].The prevalence of chronic TMD is approximately 10% [3].

Both fibromyalgia and chronic TMD patients usually experience persistent pain even in the absence of peripheral abnormalities [4-6]. This observation stimulates many investigators to research possible pathophysiological processes of these conditions intensely. Overwhelming evidence suggests that disturbance of central pain processing, namely central sensitization, is a plausible underlying mechanism for such conditions [7, 8]. Abnormalities in central pain processing have been detected in FM patients by quantitative sensory tests (QST) [9]. Features of small fiber neuropathy have been identified in FM patients, suggesting that enhanced nociceptor excitability may contribute to pain hypersensitivity and sensitization of central neural pathways [10]. Electromyography studies in FM have detected facilitated spinal nociceptive reflexes, indicating enhanced reactivity of pain pathways within the spinal cord [9]. Furthermore, patients seem to have malfunctioning endogenous pain inhibitory mechanisms, leading to amplified pain [11]. Finally, brain imaging studies have revealed augmented pain processing and altered functional connectivity within the brain pain matrix [12]. Overall, these findings indicate that FM is associated with altered nociceptive processing that mostly occurs in the central nervous system; this alteration leads to hypersensitivity, allodynia (pain initiated by normally innocuous stimuli) and thus, amplified pain.

Similarly, accumulating evidence has shown that dysregulation of central pain pathways is associated with the pathophysiology of chronic TMD. Enhanced pain sensitivity at extra-trigeminal body regions [8, 13], enhanced pain responses to low intensity repeated stimuli (temporal summation) [14], and alteration in endogenous pain modulation [15] were detected in many studies. Pressure pain thresholds of chronic TMD patients in the extra-trigeminal area are significantly lower than those of pain-free controls [16, 17]. Correspondingly, a recent systematic review and meta-analysis concluded that TMD patients had decreased pressure pain thresholds in both trigeminal and remote regions when compared to asymptomatic subjects [18].

In terms of pain location, TMD is classified as a regional pain syndrome with pain limited to orofacial regions [19], whereas widespread pain characterizes FM. According to the American College of Rheumatology Preliminary Diagnostic Criteria 2010 (ACR 2010), FM diagnosis is entertained if the following three conditions are met. First, the Widespread Pain Index (WPI) is ≥ 7 and the Symptom Severity Score (SS) ≥ 5, or the WPI is 3–6 and the SS ≥ 9. Second, symptoms have been present at a similar level for at least 3 months. Lastly, the patient does not have a disorder that would otherwise explain the pain [6]. On the other hand, TMD is diagnosed by the combination of symptom reports and validated clinical examination in the area of temporomandibular joint (TMJ) and masticatory musculature following Diagnostic Criteria of TMD (DC/TMD) [20].

FM and TMD frequently co-occur [21, 22]. Fibromyalgia patients are 31 times more likely to fulfill a diagnosis of facial muscle pain than those without the condition [23], and often report myofascial pain [23, 24]. The prevalence of TMD in FM ranges between 59.37% and 93.7% [25], while the prevalence of FM in patients with TMD was approximately 10 to 52 percent [21, 22]. Chronic TMD patients often describe widespread pain in remote regions [26]. Psychosocial factors

such as stress, depression, and anxiety have been reported as essential cofactors in both pain conditions [22, 27-31].

In summary, fibromyalgia and chronic TMD share some common features including presumed pathophysiology, contributing factors, and overlapping pain location. Growing evidence consistently suggests that central sensitization is potentially an underlying cause of altered central pain processing in both conditions.

1.2 MEASUREMENT OF CENTRAL SENSITIZATION

The manifestation of central sensitization is challenging to evaluate based solely on clinical examination. It is essential to have a tool that can quantify the severity and extent of central sensitization [32]. Quantitative sensory testing (QST) is a non-invasive method used to measure large (Aβ) and small nerve fiber function (Aδ and C fiber), including the corresponding central pathway [33]. It is a well-recognized diagnostic tool to assess somatosensory changes used by both clinicians and researchers for decades [34]. Besides neuropathic conditions, QST has been widely used to study somatosensory profiles in non-neuropathic conditions such as arthritis, myofascial pain, and fibromyalgia [35-38]. According to the German Research Network (DFNS), a comprehensive QST battery consists of thermal detection and pain thresholds, tactile detection threshold (MDT), mechanical pain threshold (MPT), mechanical pain sensitivity (MPS), dynamic mechanical allodynia (DMA), wind-up ratio (WUR), vibration detection threshold (VDT), and pressure pain threshold (PPT). Intra-examiner (test-retest) and inter-examiner reliability for intraoral and facial QST were evaluated in previous studies [39, 40]. QST is adequately reliable to investigate the mechanisms of various pain disorders in trigeminal and extra-trigeminal regions [39-43].

Central sensitization can also be assessed via the nociceptive flexion reflex (NFR), which is an objective tool that primarily explores spinal processes [44-46]. NFR is believed to be a polysynaptic reflex that includes contributions from A-delta and C nociceptive afferent fibers, interneurons, and alpha motor neurons [45]. Nociceptive flexion reflex threshold (NFRT), the least electrical intensity to initiate NFR, correlates with subjective pain threshold [47-49]. This mode of measurement allows researchers and clinicians to study central pain processing objectively. NFR has been used to measure CS in various chronic pain conditions such as fibromyalgia, irritable bowel syndromes, and whiplash-associated disorders [46, 50-53].

Electrical pain threshold (EPT) is another modality to evaluate the level of excitability of central pain pathways. The rationale is that pain hypersensitivity detected after electrical stimulation at a non-pathological area could be a consequence of an alteration in central processing [54]. Previous studies demostrated good-to-excellent reliability of EPT in both chronic pain patients and normal controls [55, 56] . Normative values of both NFRT and EPT were also established in pain free individuals [57].

1.3 IMMUNE REGULATION AND SENSITIZATION

There is strong preclinical evidence for a role of the immune system in the regulation of pain sensitivity; immune cells sensitize peripheral nociceptive neurons, and activation of glial cells leads to sensitization of central pain pathways via the release of pro-inflammatory cytokines and trophic factors [58].

1.3.1 *Immune regulation and peripheral sensitization*

When tissues are injured, an action potential is generated at the nociceptors and propagates not only to the central terminal of the neuron, but also the peripheral terminal of

primary afferent neurons. The activation of the peripheral terminal neurons consequently causes release of several neuropeptides such as substance P (SP), calcitonin gene-related peptides (CGRP), somatostatin and vasoactive intestinal peptide (VIP). These mediators then increase vascular permeability and consequent edema, which facilitates immune cell migration (including mast cells, macrophages, basophils, platelets, neutrophils, endothelial cells) to the site of injury. Recruited immune cells produce varieties of inflammatory mediators consisting of histamine, prostaglandins, thromboxane, leukotrienes, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) [59] Once these accumulated inflammatory molecules (also called inflammatory soup) are recognized by specific receptors at the primary afferent's membrane, they initiate action potential firings via the direct opening of sodium, calcium and potassium channels. In addition, some inflammatory mediators bind to peripheral afferent neurons through specific G-protein coupled receptors (GPCRs), and trigger a cascade of intracellular changes.[60, 61] As a result, the thresholds of Aδ and C nociceptors are reduced, thereby increasing the sensitivity and excitability of the nociceptors.[59] This phenomenon is defined as peripheral sensitization, which contributes to allodynia, and primary hyperalgesia (an enhanced response to noxious stimuli at the site of injury).

However, dynamic tactile allodynia, temporal summation of pain and secondary hyperalgesia (pain initiated by noxious stimuli beyond the site of injury) cannot be explained by peripheral sensitization. Evidence outlined in the following section demonstrates that peripheral sensitization, particularly via C fiber nociceptors, can induce secondary changes in spinal cord dorsal horn neurons leading to central sensitization.

1.3.2 *Immune regulation and central sensitization*

Glial cells (known as neuroglia) including microglia, oligodendrocytes, and astrocytes are non-neuronal cells functioning in the central nervous system (brain and spinal cord). Glia take up, metabolize, respond to, and release neurotransmitters and modulators, control the microenvironment that surrounds neurons, support communication, and play a number of roles in CNS homeostasis [62]. Under hazardous circumstances such as injury of the central nervous system, microbial invasion, and some pain states, microglia and astrocytes are activated [63]. Neuronal cells release neurotransmitters including substance P, glutamate, BDNF, chemokines and ATP. These mediators activate neuroglia thereby leading to the releases of proinflammatory cytokines such as IL1β, IL 6, and TNF α from the glial cell. These cytokines then further increase the discharge of excitatory amino acids and substance P from the Aδ and C afferents that synapse in the dorsal horn. This phenomenon consequently enhances the hyperexcitability of the dorsal horn neurons, leading to central sensitization [64].

In summary, the communication between neuronal and non-neuronal immunocompetent cells plays a vital role in the maintenance of homeostasis within both peripheral and central nervous system components, but under a certain condition, this bidirectional relationship can contribute to neuropathology such as the transition from acute to chronic pain [65].

1.4 IMMUNE DYSREGULATION AND CENTRAL SENSITIZATION

A plethora of evidence from animal studies has shown that the activation of non-neuronal cells located in central nervous systems (brain and spinal cord), namely glial cells or neuroglia contributes to the initiation and progression of chronic pain [63, 66-72]. Despite these exciting discoveries, the concept cannot be confirmed by clinical research as it is impossible to study glia

activity in human pain states. Therefore, the pathological basis of chronic pain in relation to neuroglia dysregulation remains unclear in humans [72].

The role of immune dysregulation and central sensitization has not been studied in chronic TMD patients. Studies have been conducted in FM patients. The degree of different types of cytokine production in FM patients compared to healthy controls has been intensely investigated [73-75]. According to a recent systematic review with meta-analysis of cytokines in fibromyalgia syndrome, variable results were reported [74]. While some studies demonstrated a higher level of cytokine production in FM patients, others revealed contradictory results [74]. Additionally, numerous studies reported no difference for cytokine expression between FM patients as compared to controls [76-81]. Small-fiber neuropathy associated with immune dysregulations has been detected in FM patients [10, 82, 83]. However, it is unclear which mechanisms control the interaction between the immune system and pain modulation in FM. One reason is the varying outcomes of studies investigating blood levels of cytokines, which is at least in part a consequence of methodological issues and inadequate research design. Authors of systematic reviews, therefore, have called for more hypothesis-based and mechanistic studies [73, 74].

1.5 TOLL-LIKE RECEPTORS IN CHRONIC PAIN

Toll-like receptors (TLR) are pattern recognition receptors that have a significant role in innate immunity in both peripheral and central immune systems. In the periphery, TLRs are expressed on antigen-presenting cells including monocytes and macrophages [84]. Similarly, TLRs have been identified in central nervous system neurons and glial subtypes including microglia, astrocytes, and oligodendrocytes [85].

TLRs are capable of sensing danger-associated molecular patterns (DAMPs), also called self-molecules, or pathogen-associated molecular patterns (PAMPs) both endogenously and exogenously. Once TLRs specifically interact with a certain area of the molecules, pro-inflammatory cytokines are released. Examples of exogenous TLR agonists include lipopolysaccharide recognized by a TLR 4 receptor, single-stranded viral RNA (by TLR7/8 receptor) and saturated fatty acid (by TLR2 and TLR 4 receptor) [86]. To date, TLRs 1 to13 have been identified in humans [87].

TLR activation has consistently been associated with pain hypersensitivity in models of nociceptive and neuropathic pain [86]. Interestingly, preclinical research found that blocking TLRs genetically or pharmacologically can reduce microglia activation, resulting in the reduction of pro-inflammatory cytokine levels and thus reduction in experimentally-induced neuropathic pain in mice [84].

Growing evidence suggests that the dysregulation of innate immunity in the CNS may be a potential driving force of chronic pain. An exaggeration of glia response via the production of pro-inflammatory mediators such as IL1β contributes considerably to increased neuron responsiveness, potentially leading to central sensitization [72]. These studies, however, were conducted in animals. It is impossible to directly investigate TLR responsiveness of glial cells in chronic pain sufferers. Hence, few studies have looked at TLR responsiveness in PBMCs, assuming that TLRs have similar functions in CNS innate immune cells and the periphery. Impressive work from Kwok and colleagues showed that increased IL1β expression was significantly higher in PBMCs from chronic pain sufferers (on, and not on opioids) compared with pain-free controls for TLR2 ($P<0.001$), TLR4 ($P= 0.002$) and TLR7 ($P= 0.005$) agonists. Those data demonstrated that PBMCs from chronic pain sufferers were more responsive to TLR

agonists compared with controls, suggesting that peripheral blood cells may have the potential to become a source of biomarkers for chronic pain [96, 97].

1.6 G-PROTEIN COUPLED RECEPTOR KINASE 2 IN CHRONIC PAIN

GRK2 is a homeostatic regulator of G protein-coupled receptors including chemokine receptors [88]. In addition, GRK2 directly interacts with inflammatory signaling pathways involving extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinases [89]. Activation of GRK2 reduces the sensitivity of immune cells to chemokines, thus preventing exaggerated responses to these endogenous inflammatory mediators [90]. GRK2 is expressed in microglial cells, blood monocytes and peripheral macrophages [91]. Reduced levels of GRK2 in peripheral blood mononuclear cells (PBMCs) have been detected in patients with rheumatoid arthritis and multiple sclerosis, supporting the hypothesis that GRK2 is a biomarker for the endogenous control of inflammatory disease processes [92, 93]. Downregulation of GRK2 in microglia and macrophages in experimental models of neuropathic and inflammatory pain produces central sensitization, and transforms acute into chronic hyperalgesia [88, 89, 91]. A low level of GRK2 expression or function may therefore be an important mechanism underlying long-term hyperalgesia.

1.7 ASSOCIATION BETWEEN GRK 2 AND TOLL-LIKE RECEPTORS

Both Toll-like receptors (TLRs) and G-protein-coupled receptor kinase 2 (GRK2) play important roles in regulating immune responses, as mentioned in sections 1.5 and1.6. There is evidence that TLR4 activation suppresses the expression of GRK2 via the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) [94, 95]. TLR stimulation increases the expression of IL1β in peripheral monocytes of patients with chronic pain [96, 97]. Thus,

molecular and functional changes in innate immune activity that involve glial cells and lead to pain hypersensitivity may be associated with similar changes in PBMCs.

Chapter 2. HYPOTHESES

Given the functional similarity between TLR signaling of immune cells in the periphery and CNS, we hypothesized that molecular and functional changes in innate immune activity that involve glial cells and lead to pain hypersensitivity might be associated with similar changes in peripheral blood cells.

The responsiveness of TLR 2, 4, 7 ligands have been studied in chronic pain populations [96], but not specifically in fibromyalgia and TMD patients who share some similar features. Both conditions are characterized by chronic pain that is not explained by tissue lesions.
We proposed a model to explain central sensitization in FM and chronic TMD that involves dysregulation of innate immune activity. If the hypothesis was confirmed, TLR activity in peripheral blood cells might reflect a systemic shift in the reactivity of the innate immune system, providing a clinically accessible marker of the responsiveness of glial cells.

Our hypotheses were: 1) In comparison to normal controls, patients with FM and chronic TMD display lower basal GRK2 levels in PMBCs. 2) Basal GRK2 levels are correlated negatively with indices of central sensitization.3) In comparison to normal pain-free controls, patients with FM and TMD display higher IL-1β production after TLRs stimulation. 4) IL1β levels are positively correlated with indices of central sensitization.

For the purpose of this pilot study, we concentrated on IL-1β as a biochemical outcome measure of leukocyte reactivity. IL-1β was chosen among cytokines because it has been shown to be elevated after TLRs 2, 4, and 7 stimulation in human studies [96, 97].

Our original plan was to compare the level of IL1β expression following TLR 2, 4, 7 stimulation among FM patients, chronic TMD patients, and pain-free controls. This thesis will be focusing on delivering the preliminary data of TLR 2 responsiveness because toll-like receptor 2

responded best to all concentrations of TLR 2 agonist. This toll-like receptor also provided the most notable trend as compared to other TLR agonists (TLR 4, TLR7).

Chapter 3. MATERIALS AND METHODS

3.1 STUDY DESIGN AND OVERVIEW

We conducted a case-control study to assess pain sensitivity, and IL1β levels in two groups of chronic pain patients and controls. The study involved completion of questionnaires and tests of pain sensitivity, as well as a blood draw. All these procedures were completed at the Oral Medicine Clinical Service in one visit. Ethical approval was obtained from the Human Subjects Division (HSD), Office of Research at University of Washington. Participants received $100 as compensation upon study completion.

This project is the first report of the pilot study (G-protein-couple receptor kinase 2 and toll-like receptors as regulators of central sensitization in fibromyalgia and chronic temporomandibular disorders). According to the original sample size calculation based on previous studies [92], we planned to complete 18 subjects per group (18 controls, 18 fibromyalgia, 18 TMD). However, mitigating circumstances including limited budgets, challenges of recruiting chronic pain subjects without opioid usage, and time constraint, produced preliminary analyses utilizing the data collected from 18 controls, 11 TMD subjects, and 9 fibromyalgia subjects.

The main outcomes were

1) Level of central sensitization (details are discussed in 3.4.1)

2) Basal IL1β level

3) IL1β level after toll-like receptors 2 stimulation

4) Basal GRK 2 level on PBMCs

For participants of reproductive age, both blood draw and assessment of central sensitization were performed 1-5 days after the beginning of the menstrual period in order to account for possible variations in TLR activity and pain sensitivity according to the menstrual cycle.

3.2 SUBJECT ENROLLMENT

3.2.1 *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.

191 potential TMD subjects who came to Oral Medicine Clinical Services between December 2016 and December 2018 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. Twenty 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.[5] (Questionnaires & criteria in Appendices).
7. Only potential TMD participants who met the diagnosis of DC TMD [20] 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.

3.2.2 *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)[98]

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.

3.3 STUDY SCHEDULE

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 [99].
- Generalized anxiety disorder – 7 (GAD-7) for the assessment of anxiety [100].
- Fibromyalgia survey score for the quantification of fibromyalgia symptoms [5].
- The diagnostic criteria for temporomandibular disorders – Symptom questionnaire [20].

- Jaw functional limitation scale - 20 (JFLS-20) [101].

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 by one investigator to confirm diagnosis in combination with history, following DC/TMD diagnostic criteria.[20]

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 by one investigator 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)

3.4 METHOD

3.4.1 Assessment of Central Sensitization

7.4.1.1 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)

7.4.1.2 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.

7.4.1.3 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.

7.4.1.4 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 μV 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).

3.4.2 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 by one experienced lab technician at Harborview Research and Training Center. 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 IL-1β 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.

3.5 STATISTICAL ANALYSIS

Descriptive statistics were computed for all variables. Percentages were calculated for discrete variables and means and standard deviations were computed for continuous variables. Age is an essential factor for pain sensitivity and TLR responses. We therefore planned to match patients' age ±5 years with the age of controls. Since we did partial analysis based on collected data (17 controls, 11 TMD, 9 FM), there were small differences in ages between the patients and controls. This difference, however, was not statistically significant ($p = .163$)

Remark: As the baseline GRK 2 level is currently pending evaluation by Western blot, this statistic analysis focused on IL1β expression at baseline and after TLR 2 stimulation.

To test the primary hypothesis that following TLR stimulation, FM and TMD patients will have higher concentrations of IL-1β than pain-free controls, we performed a repeated measures ANOVA, with TLR 2 agonist (Pam3CSK4) at each concentration; 10 ng/ml, 100ng/ml, 1000 ng/ml as the within-subject factor, and group as the between-subjects factor. The dependent variable was post-stimulation IL-1β concentration (pg/ml).

To test the secondary hypothesis that IL-1β levels are correlated positively with indices of central sensitization, we performed Pearson correlations: IL-1β levels vs. pressure pain threshold, IL-1β levels vs. windup ratio, IL-1β levels vs. nociceptive reflex threshold, IL-1β levels vs. electrical pain threshold, and IL-1β levels vs. pain area.

Chapter 4. RESULTS

4.1 STUDY SAMPLE

TMD: of 191 potential subjects, 155 patients were excluded due to the following reasons: 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) Some subjects were excluded for multiple reasons.

Among 36 qualified TMD subjects, 14 subjects dropped out from the study, five met criteria for a fibromyalgia diagnosis, six subjects are pending testing as of this writing*, and thus 11 TMD subjects were tested and included in the analysis.

Fibromyalgia: five TMD subjects who also met the diagnosis of fibromyalgia were assigned to the fibromyalgia group. Sixteen potentials subjects were additionally screened. Of those, 12 were excluded from the study due to opioid usage (n = 10) or autoimmune condition (n = 2). Nine fibromyalgia patients were therefore tested and included in the analysis.

Control: We approached a total of 24 potential controls. Of those, four were excluded from the study due to autoimmune disease, tension-type headache, migraine or using systemic hormone replacement therapy. We tested 18 control subjects during the study period. One of the tested controls was later excluded due to migraine diagnosis, which was reported after testing. Two subjects are pending testing as of this writing*.

*These subjects will be tested and included in the future analysis when we finish this pilot study.

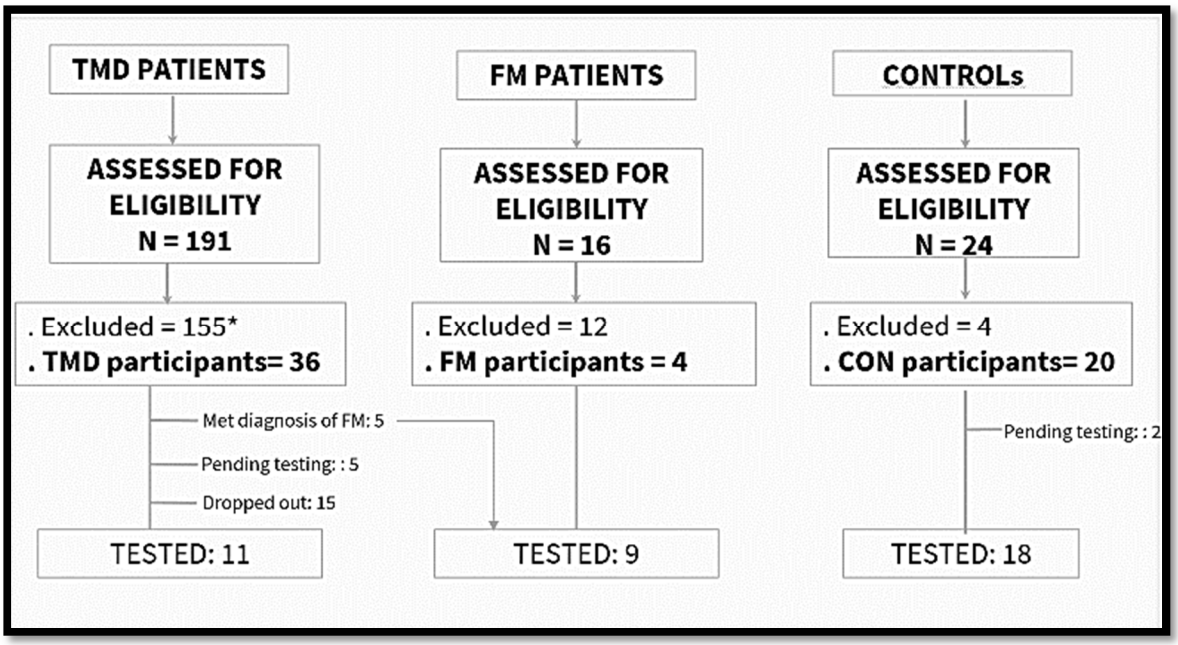

Figure 1. A flowchart demonstrating subject enrollment.

4.2 DEMOGRAPHIC, PAIN-RELATED, AND PSYCHOLOGICAL DATA

18 pain-free controls, 11 TMD and 9 FM patients were tested. One of the controls was excluded due to migraine diagnosis reported after the testing visit. Demographic data, pain-related, and psychological variables are reported in Table 1.

Regarding medication usage, two out of 17 controls were receiving aspirin, one acetaminophen, and two controls hormone replacement therapy (Intrauterine device, Mirena ®). One out of 11 TMD patients was receiving a selective serotonin-reuptake inhibitor (SSRI), one a serotonin-norepinephrine reuptake inhibitor (SNRI), five muscle relaxants (cyclobenzaprine, benzodiazepine), three anticonvulsants (gabapentin), two non-steroidal anti-inflammatory drugs (NSAIDs), and two topical hormone replacement therapy (Estradiol cream, Estradiol vaginal

ring). One out of nine FM patients was receiving tricyclic antidepressants (TCA), one a selective serotonin-reuptake inhibitor (SSRI), two patients serotonin-norepinephrine reuptake inhibitors (SNRI), one an anxiolytic, three patients muscle relaxants, two gabapentin, two non-steroidal anti-inflammatory drugs (NSAIDs), and three analgesics. None of the FM patients were using any form of hormone replacement therapy.

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.

Table 1 Demographic, pain-related and psychological variables

	Control (n=17) Median(Q1-Q3)	TMD (n=11) Median(Q1-Q3)	FM (n=9) Median(Q1-Q3)
Age (years)	56 (37-62)	60 (38-66)	65 (52-70)
BMI (kg/m2)	24 (20-26)	23 (19-25)	28 (26-34)
Race % (n)*			
Asian/Pacific	41.2% (7)*	9% (1)	11.1% (1)
Hispanic	11.8% (2)*	9% (1)	11.1% (1)
Caucasian	29.4% (5)*	81.8% (9)	55.5% (5)
African American	0	0	11.1% (1)
≥2 identified ethnicities	17.6% (3)*	0	11.1% (1)
Area of pain (pixels)	0	76440 (27840, 114474)	325816 (226946,1745348)
Pain duration (years)	0	5 (1,20)	20 (5,32)
Pain intensity at the testing day (0-10)	0	4 (3,5)	5 (4,6)
Average pain in the past week (0-10)	0	5 (3,6)	6(4,7)
Pain interference (0-10)			
General activity	n/a	4 (2,6)	6 (3,7)
Enjoyment of life	n/a	4 (2,8)	5 (3,9)
Falling asleep	n/a	3 (2,5)	7 (2,9)
Staying asleep	n/a	3 (2,4)	7 (3,9)
PHQ-9	0 (0,1.5)	4 (2,6)	12 (5,18)
GAD-7	0 (0,1)	1 (0, 6)	6 (1,14)
JFLS-20	0	61 (38, 95)	31 (5,72.5)

4.3 PAIN-RELATED MEASURES

4.3.1 *Pressure Pain Threshold*

One-way ANOVA analysis of PPT demonstrated significant differences comparing pain-free subjects, the TMD patient group and the FM patient group for both masseter region ($p<.001$), and scapula area ($p<.001$), but not for the site of the second toe ($p = 0.581$). (See

Figure 2 and Table 3.) For the masseter region, the Bonferroni post hoc test showed significant differences between controls and TMD ($p<0.001$), and controls and FM subjects ($p<0.001$). Likewise, there were significant differences between controls and TMD patients ($p<.001$), and controls and FM patients ($p<.01$) for PPT of the scapula region. PPT of TMD and FM patient groups did not differ significantly for the masseter or scapular region ($p>0.05$).

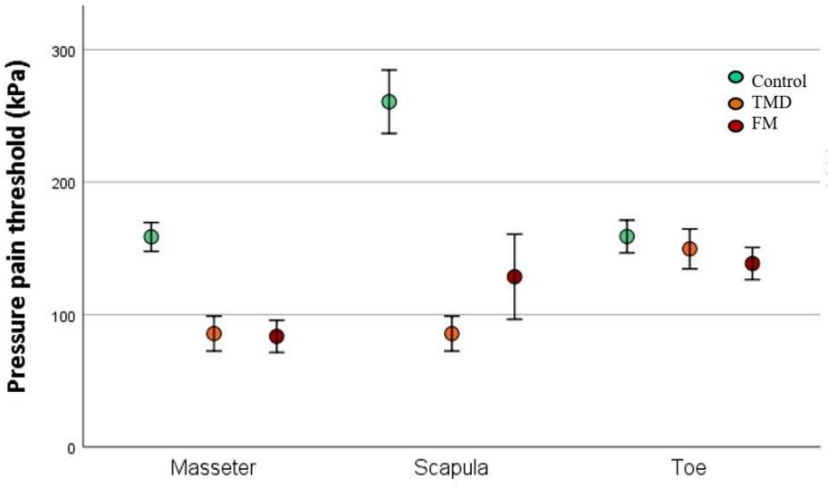

Figure 2. Mean pressure pain threshold (PPT) ± Standard error of the mean

4.3.2 *Wind up ratio (WUR)*

ANOVA of the WUR on each body area (masseter, scapula, dorsum of the foot) showed no significant differences among pain-free controls, TMD patients, and FM patients; p-values were 0.509, 0.304, and 0.693, respectively (See Table 3 for summary statistics)

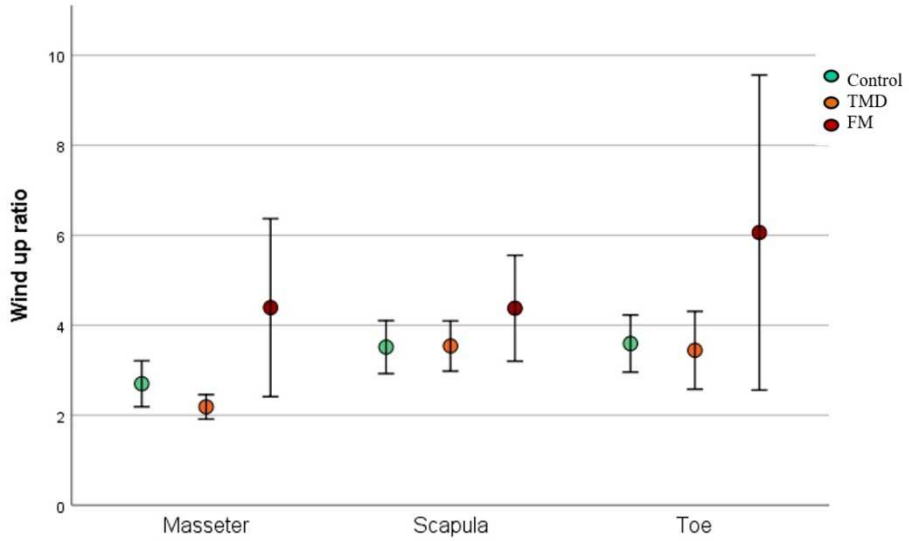

Figure 3. Mean wind up ratio (WUR) ± standard error of the mean.

4.3.3 *Nociceptive Reflex Threshold and Electrical Pain Threshold*

Nociceptive flexion reflex threshold was significantly different among controls, patients with TMD, and patients with FM ($p<0.01$, see Figure 4). The Bonferroni post hoc test indicated that the TMD group demonstrated significantly higher nociceptive flexion reflex threshold compared to the control group ($p < 0.05$). Similarly, the FM patients group had significantly higher nociceptive flexion reflex threshold when compared to the control group ($p < 0.05$). There was an outlier in the FM group where NFR was detected at her second visit. We were not able to initiate her reflex within the first appointment visit. After excluding this patient's data, the difference of NFRT between FM subjects and controls was not statistically significant. No statistical difference was found between the cases (TMD, FM).

Regarding EPT, there were no statistically significant differences in electrical pain threshold among three groups ($p = 0.543$) (see Figure 5)

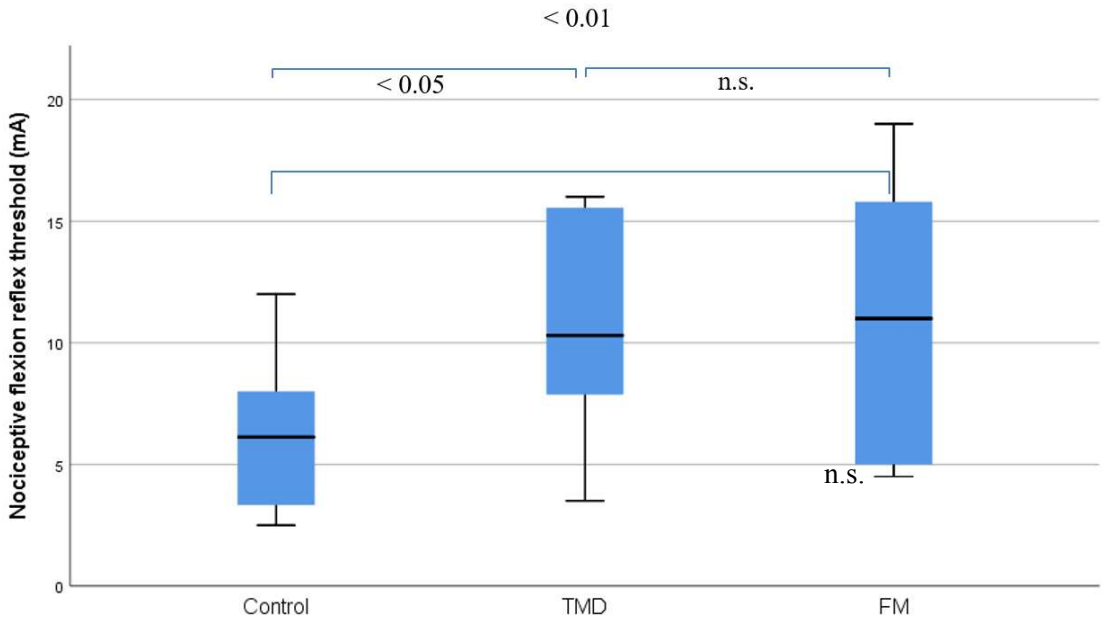

Figure 4. Nociceptive flexion reflex threshold in Controls, TMD patients, and FM patients.

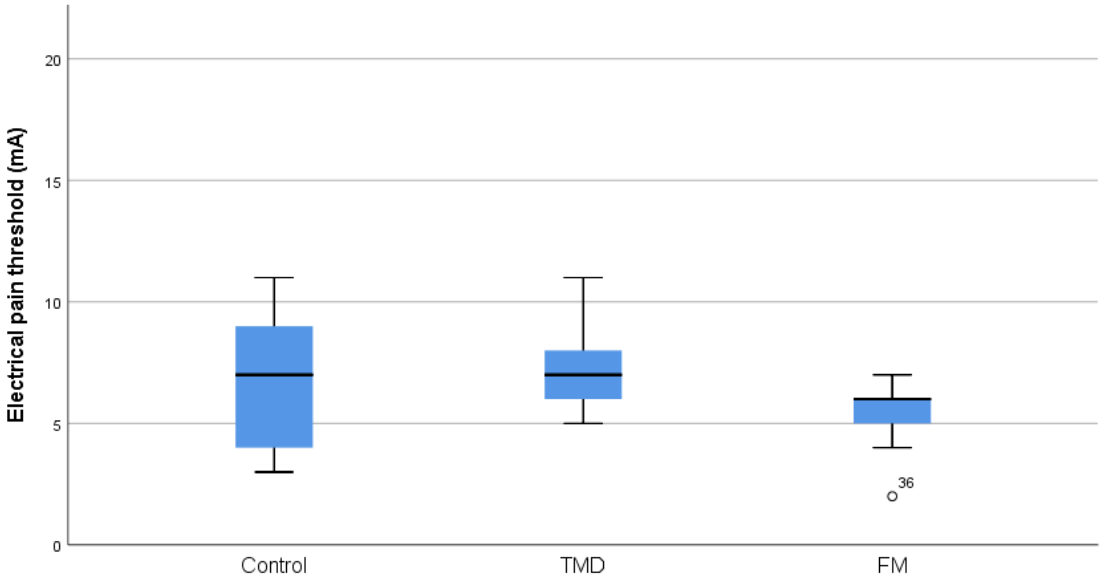

Figure 5. Electrical pain threshold (EPT) in controls, TMD patients, and FM patients.

4.3.4 *Anatomical distribution of pain*

Regarding pain area, a significant difference was found between pain-free participants, TMD participants, and FM participants ($p<0.001$). According to the Bonferroni post hoc test, the number of pixels among TMD patients and FM patients differed significantly with the FM group reporting a larger affected area ($p<.01$). Similarly, the FM group had a significantly higher number of pixels than controls ($p<.001$). (See Figure 6 for boxplot graph of pain area).

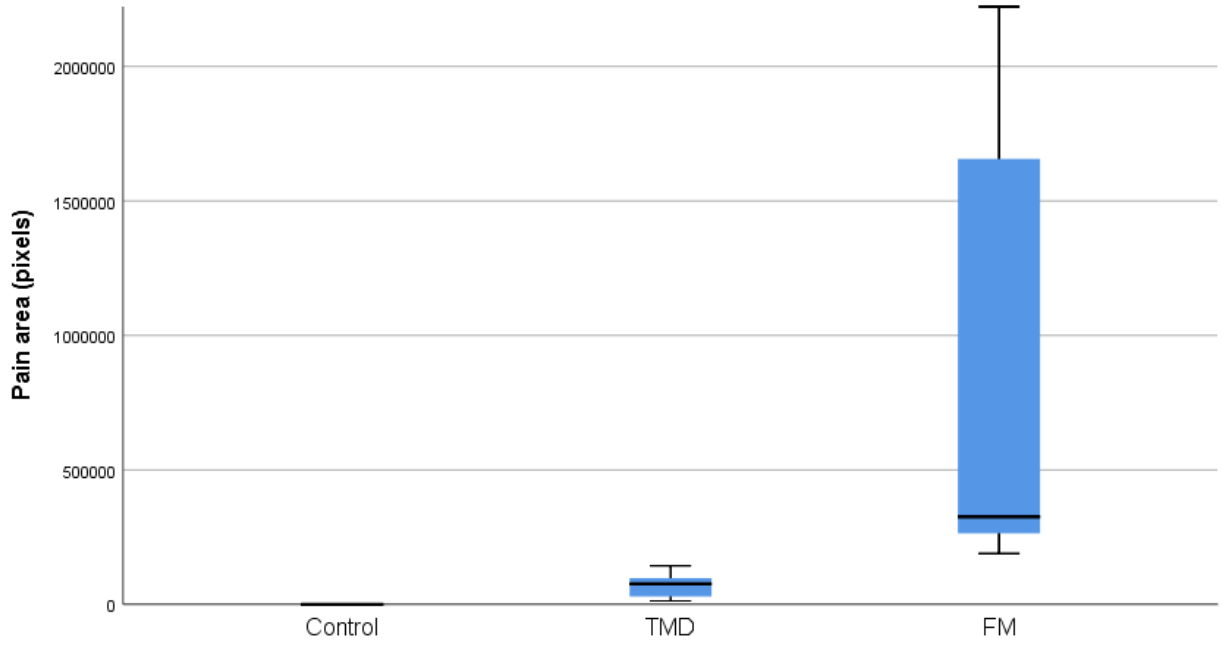

Figure 6. Pain area in controls, TMD patients, and FM patients.

Variables	Control					TMD					FM					P value
	$\bar{x}$	SD	Median	Q1	Q3	$\bar{x}$	SD	Median	Q1	Q3	$\bar{x}$	SD	Median	Q1	Q3	
PPT (kPa)																
2 nd toe	159	51	147	129	170	150	50	134	110	200	139	36	144	120	171	0.581
Masseter	159	45	150	133	190	86	44	77	57	108	84	37	86	62	96	< 0.001
Scapula	261	99	235	210	356	86	44	77	57	108	129	96	104	60	152	< 0.001
WUR																
2 nd toe	4	3	3	2	4	3	3	2	1	6	6	11	2	2	4	0.509
Masseter	3	2	2	1	3	2	1	2	2	3	4	6	3	2	3	0.304
Scapula	4	2	2	2	4	4	2	3	2	5	4	4	3	2	6	0.693
NFRT (mA)	6	3	6	3	8	11	5	10	7	16	11	6	11	5	16	0.006
EPT (mA)	9	13	7	4	9	7	2	7	6	8	5	2	6	5	6	0.543
Pain area (Pixel)	0	0	0	0	0	70917	46624	76440	27840	114474	918914	962541	325816	264154	1656236	< 0.001

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

Abbreviations

PPT: Pressure pain threshold; 2nd toe: second toe; WUR: wind up ratio; NFR: nociceptive reflex threshold; EPT: electrical pain threshold

$\bar{x}$: Arithmetic mean; SD: standard deviation; Q1: first quartile; Q3 third quartile

4.4 BASAL GRK 2 LEVEL

We are currently conducting the blood analysis to evaluate GRK 2 level from all participants. The results, therefore, will be reported in the future.

4.5 IL1B LEVEL FOLLOWING TLR 2 STIMULATION

We stimulated TLR 2 with three different concentrations of TLR 2 agonist (Pam3CYSK4); 10 ng/ml, 100ng/ml, 1000 ng/ml. All detailed data are provided in Table 3.1. Repeated measures ANOVA analysis (Table 2.2) shows that there was a significant difference of IL1β expression for different concentrations of TLR 2 agonist ($p<.001$). Pam3CYSK4 at the concentration of 1000 ng/ml provided the largest amount of the released cytokine following TLR

2 stimulation. When analyzing for interaction between all levels of Pam3CYSK4 concentration, and case status (control, TMD, FM), no statistically significant difference was observed.

Table 3.1 Changes of IL1β level after TLR 2 stimulation

Changes of IL1β levels after TLR 2 stimulation (pg/ml)																		
TLR 2 agonist	10 ng/ml						100 ng/ml						1000 ng/ml					
	$\bar{x}$	SD	Median	Q1	Q3	p	$\bar{x}$	SD	Median	Q1	Q3	p	$\bar{x}$	SD	Median	Q1	Q3	p
Control	27.0	17.8	27.9	10.1	42.4		91.3	64.3	74.6	43.5	133.9		182.7	137.2	137.5	68.0	295.2	
TMD	15.1	12.8	12.2	2.2	28.0	0.07	51.3	40.7	34.1	22.5	77.1	0.15	86.9	76.6	50.7	28.3	140.5	0.11
FM	13.9	13.1	10.8	5.4	16.7		57.3	52.8	33.9	22.5	79.3		107.3	125.8	51.2	35.1	132.7	

Table 3.2 Test of within-subjects effects (Pam3CYSK4).

Source	Type III Sum of Squares	df	Mean Square	F	p-value
Intercept	496709.4	1	496709.4	44.1	0.000
Case status	54058.6	2	27029.3	2.4	0.106
Error	359780.8	32	11243.2		

4.6 CORRELATIONS BETWEEN CENTRAL SENSITIZATION MEASURES AND IL1B LEVELS

A significant correlation was identified between IL1β level released after TLR 2 stimulation (Pam3CYSK4 1000 ng/ml) and nociceptive flexion reflex threshold ($r = -.386$, $P<.05$). Figure 7 illustrates the negative correlation between the parameters.

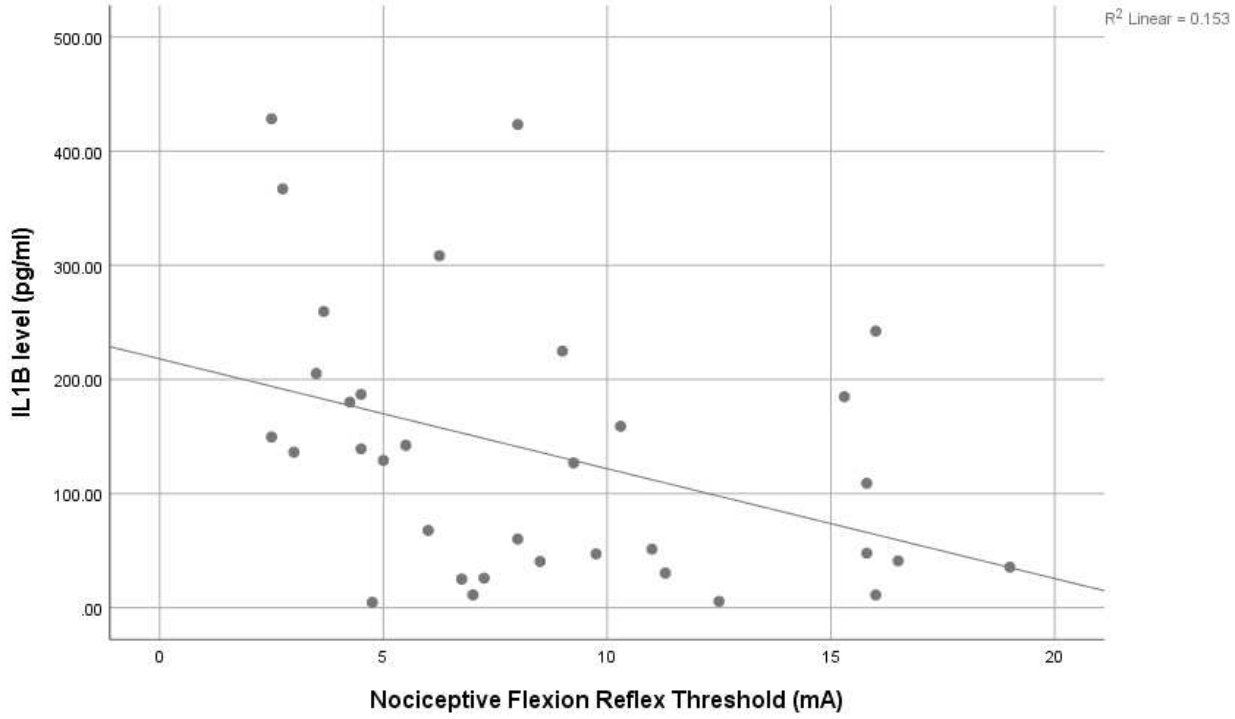

Figure 7. Scatter plots illustrating the relationship between IL1β level and the nociceptive flexion reflex threshold (NFRT).

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

Table 4. Pearson correlation between measures of CS and IL1β expression after TLR 2 stimulation (by Pam3CYSK4 1000ng/ml)

Pearson correlations	
Measures of CS	Correlation with IL1β level
PPT toe (kPa)	-0.167
PPT masseter (kPa)	0.080
PPT scapula (kPa)	0.192
WUR toe	-0.032
WUR masseter	-0.033
WUR scapula	-0.025
Nociceptive flexion reflex threshold (mA)	-0.386
Electrical pain threshold (mA)	-0.020
Pain area (No. of pixels)	-0.199

*Correlation is significant at the 0.05 level (2-tailed)

Abbreviations
CS: Central sensitization
PPT: Pressure pain threshold (Kilopascal, kPa)
WUR: Wind up ratio
No. of pixels: numbers of pixels

Chapter 5. DISCUSSION

5.1 PAIN RELATED MEASURES

5.1.1 *Pressure pain threshold*

PPT was significantly higher in pain-free participants compared to the case groups over the area of masseter and scapula. This finding was in line with many studies [13, 18, 102, 103]. Interestingly, no significant differences were detected between TMD and FM in any regions. This could be explained by the coexistence of TMD in FM patients; 8 out of 9 FM subjects met the diagnosis of masticatory myofascial pain with referral.

5.1.2 *Wind up ratio*

We were not able to detect significant differences in wind-up ratio among the three groups. This might be related to the level of reliability of the test and/or to insufficient sample size. Work from Geber and coworkers showed moderate reliability for WUR when performed in a heterogeneous pain population [104]. Similarly, the reliability of WUR was only fair in a study using the same QST protocol in chronic orofacial pain patients [40]. Previous studies attributed the findings to the failure of reporting pain by participants even at maximum intensity of stimuli, and intolerable pain from pinprick stimuli. However, our study was able to measure WUR from all participants. Many participants had difficulty rating pain using a 0-100 scale. Some chose to rate pain within 0-10, while others reported as instructed. Two FM patients developed tingling sensation and numbness during testing sessions over the scapula and toe. The test was paused and continued after the sensation had resolved.

5.1.3 *Nociceptive flexion reflex threshold*

Regarding NFRT, the FM group had significantly higher nociceptive flexion reflex threshold when compared to the control group. The result is not in accordance with other studies [50]. However, the difference was not statistically significant after excluding a FM outlier who completed NFR testing on two weeks apart via manual mode (stimuli was incrementally increased by 1 mA until the NFR was evoked). The patient displayed substantially elevated scores on several dimensions of the PHQ-9, GAD-7, widespread pain index, symptom severity score, and pain interference. The difficulty in determining her reflex is consistent with the finding from previous studies that NFR might be impaired by severe symptoms of depression and fibromyalgia, potentially due to desensitization of NFR pathways [105, 106]. Also, the fact that her NFRT was more than twice her EPT suggested that the reflex could be a false measurement due to improper placement of electrodes. The present study also encountered a number of difficulties detecting the reflex in the fibromyalgia group with randomization mode. Thus, electrical stimuli were delivered incrementally by an investigator to be able to detect NFRT in four out of nine FM subjects.

Our experiment provided novel data regarding NFRT in women who had chronic TMD. The experiment revealed that TMD subjects had a significantly higher level of NFRT than controls. This finding was not in accord with the theory that hyperexcitability of the spinal cord in chronic pain patients should result in lower NFRT when compared to the pain-free group. This contradictory result may be attributed to pain-modulating compounds such as acetaminophen, NSAIDs, muscle relaxants, and antidepressants [107-

109] which could heighten NFRT. This may have influenced our results, as we did not attempt to discontinue participants from medication usage prior to the testing visit.

5.1.4 *Electrical pain threshold*

No studies have attempted to compare electrical pain threshold (EPT) among FM patients, TMD patients, and healthy controls to date. From a review of the literature we found that researchers have utilized the lower extremity for evaluating EPT in fibromyalgia patients, whereas others measured EPT over the trigeminal area in TMD patients. The present study indicated that there was no significant difference among the groups. A non-significant trend suggested that pain threshold to electrical stimuli was lowest in the FM group, followed by the TMD group, and finally controls. This finding could be attributed to insufficient sample size, or the methodology may not be sensitive enough to detect generalized hypersensitivity. Banic et al. assessed pain threshold to electrical stimuli, and spinal reflex threshold in whiplash and fibromyalgia patients as compared to healthy controls. While spinal reflex threshold was significantly lower in patients compared to controls, the difference in electrical pain threshold was only detected between fibromyalgia patients and controls, suggesting that an electrical pain threshold may be a less sensitive method for detecting central hypersensitivity than spinal reflex testing [50]. These findings were supported by a study by Lautenbacher et al. who measured EPT, PPT, and heat pain threshold over a control point and a tender point of fibromyalgia patients as compared to pain-free controls. Other measures of CS demonstrated a significant difference between the groups at both tender and control points. However, the electrical pain threshold differed between groups only at the tender point [110].

5.1.5 *Anatomical distribution of pain*

Pain distribution was significantly different among three groups; the highest number of pixels were reported by FM patients, followed by TMD patients, and pain-free controls, respectively. This is not surprising, as we aimed to allocate chronic TMD patients who met the FM diagnosis criteria to the FM patient group. It is interesting to note that 55% of FM participants had also met the diagnostic criteria of TMD. This observation was consistent with previous evidence demonstrating that TMD and FM commonly coexist [21-25].

5.2 IL1B LEVEL FOLLOWING TLR 2 STIMULATION

No statistically significant difference was identified between the IL1β expression following TLR 2 stimulation among pain-free controls, TMD and FM subjects. The non-significant trend showed that controls expressed higher levels of IL1β following TLR 2 stimulation, compared to the TMD and FM groups. This observation appeared in a homogeneous direction across three concentrations of TLR 2 agonist. These trends were, however, contradictory to the previous clinical studies from Kwok and colleagues, who demonstrated that increased IL1β expression after TLR 2 stimulation was significantly higher in chronic pain patients when compared to healthy controls [96, 97]. Based on the current knowledge, a higher level of IL1β in the control group cannot be explained and requires further investigation.

Possible explanations for our paradoxical trend may be the small sample size, different population investigated, and possible confounding factors. First, the previous studied from Kwok and colleague focused on IL1β expression following TLR stimulation in a diverse

chronic pain population; two subjects were fibromyalgia patients and nobody had a TMD diagnosis. Secondly, sex hormone level is a possible confounding factor which could alter TLR 2 response in the control group. Even though we controlled for sex and age (median ±5 years), more menopausal women were enrolled in the TMD and FM group than in the control group. Studies have shown that women lose on average 80% per year of their estrogens during the first year of menopause [111]. As estrogen modulates the immune response by upregulating inflammatory processes [112, 113], this might have produced more elevated IL1β expression following TLR 2 stimulation in the control group, when compared to the patient groups.

The lack of statistically significant differences in measurements distant from the site of the pain and in temporal summation suggest that our patients were not characterized by significant central sensitization. This may be one of the explanation for the lack of enhanced IL1β expression after TLR stimulation.

5.3 Correlations between clinical pain measures and IL1B level

We conducted analyses to evaluate whether TLR 2 agonist-induced IL1β level in leukocytes (1000ng/ml PAM3CYSK4) correlated with any of the measurements of central sensitization. A negative correlation was found between nociceptive flexion reflex threshold and level of IL1β release ($r = -.386$, $p<.05$). This finding suggested that TLR 2 responsiveness via IL1β expression may be one of the mechanisms underlying central sensitization in the spinal cord.

5.4 Study strengths and limitations

This is the first study to explore the potential role of peripheral immune response in fibromyalgia and chronic TMD. Our study had several strengths. We controlled for many

possible confounding factors which could alter both TLR responsiveness and pain-related measures (sex, age, opioid use, systemic sex hormone therapy, autoimmune diseases, neurologic diseases, migraine, etc.). TLR stimulation and pain measurements were independently conducted at the same visit by a lab technician and an investigator.

The limitations of this study include: (1) the small sample size which could compromise a number of results of the study; (2) time of blood draw and blood stimulation were not standardized across subjects. This could potentially have affected leukocyte response due to the freshness of the cells; (3) even though opioid usage in the past three months prior to testing was an exclusion criterion, participants were not asked to discontinue pain-related medications such as NSAIDs, analgesics, SSRIs or SNRIs. Some of these medications have been reported to have effects on TLR expression and some pain-related measures.

5.5 CONCLUSION

IL1β expression after TLR 2 stimulation in peripheral blood cells did not differ between FM, chronic TMD, and pain-free controls. The negative correlation between IL1β expression and spinal nociceptive reflex suggests that TLR 2 responsiveness via IL1β expression may be one of the mechanisms underlying central sensitization in the spinal cord. Because the mechanisms underlying immune dysregulation related to central sensitization are likely multiple and complex, further studies investigating the TLR responsiveness via different kinds of cytokine are needed to fully elucidate the role of TLR in fibromyalgia and TMD.

Chapter 6. REFERENCES

1. Mansfield, K.E., et al., *A systematic review and meta-analysis of the prevalence of chronic widespread pain in the general population.* Pain, 2016. 157(1): p. 55-64.
2. Wolfe, F., et al., *Fibromyalgia prevalence, somatic symptom reporting, and the dimensionality of polysymptomatic distress: results from a survey of the general population.* Arthritis Care Res, 2013. 65(5): p. 777-85.
3. LeResche, L., *Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors.* Crit Rev Oral Biol Med, 1997. 8(3): p. 291-305.
4. Auvenshine, R.C., *Temporomandibular disorders: associated features.* Dent Clin North Am, 2007. 51(1).
5. Wolfe, F., et al., *Fibromyalgia criteria and severity scales for clinical and epidemiological studies: a modification of the ACR Preliminary Diagnostic Criteria for Fibromyalgia.* J Rheumatol, 2011. 38(6): p. 1113-22.
6. Wolfe, F., et al., *The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity.* Arthritis Care Res (Hoboken), 2010. 62(5): p. 600-10.
7. Williams, D.A. and D.J. Clauw, *Understanding fibromyalgia: lessons from the broader pain research community.* J Pain, 2009. 10(8): p. 777-91.
8. Sarlani, E. and J.D. Greenspan, *Evidence for generalized hyperalgesia in temporomandibular disorders patients.* Pain, 2003. 102(3): p. 221-6.
9. Banic, B., et al., *Evidence for spinal cord hypersensitivity in chronic pain after whiplash injury and in fibromyalgia.* Pain, 2004. 107: p. 7-15.
10. Serra, J., et al., *Hyperexcitable C nociceptors in fibromyalgia.* Ann Neurol, 2014. 75(2): p. 196-208.
11. Kosek, E. and P. Hansson, *Modulatory influence on somatosensory perception from vibration and heterotopic noxious conditioning stimulation (HNCS) in fibromyalgia patients and healthy subjects.* Pain, 1997. 70(1): p. 41-51.
12. Jensen, K.B., et al., *Patients with fibromyalgia display less functional connectivity in the brain's pain inhibitory network.* Mol Pain, 2012. 8: p. 32.
13. Pfau, D.B., et al., *Somatosensory profiles in subgroups of patients with myogenic temporomandibular disorders and Fibromyalgia Syndrome.* Pain, 2009. 147(1-3): p. 72-83.
14. Maixner, W., et al., *Sensitivity of patients with painful temporomandibular disorders to experimentally evoked pain: evidence for altered temporal summation of pain.* Pain, 1998. 76: p. 71-81.
15. King, C.D., et al., *Deficiency in endogenous modulation of prolonged heat pain in patients with Irritable Bowel Syndrome and Temporomandibular Disorder.* Pain, 2009. 143(3): p. 172-8.
16. Malow, R.M., L. Grimm, and R.E. Olson, *Differences in pain perception between myofascial pain dysfunction patients and normal subjects: a signal detection analysis.* J Psychosom Res, 1980. 24(6): p. 303-9.

17. Kashima, K., et al., *Increased pain sensitivity of the upper extremities of TMD patients with myalgia to experimentally-evoked noxious stimulation: possibility of worsened endogenous opioid systems.* Cranio, 1999. 17(4): p. 241-6.
18. La Touche, R., et al., *Evidence for Central Sensitization in Patients with Temporomandibular Disorders: A Systematic Review and Meta-analysis of Observational Studies.* Pain Pract, 2018. 18(3): p. 388-409.
19. Dworkin, S.F. and L. LeResche, *Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique.* J Craniomandib Disord, 1992. 6(4): p. 301-55.
20. Schiffman, E., et al., *Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) for Clinical and Research Applications: recommendations of the International RDC/TMD Consortium Network* and Orofacial Pain Special Interest Groupdagger.* J Oral Facial Pain Headache, 2014. 28(1): p. 6-27.
21. Leblebici, B., et al., *Coexistence of fibromyalgia, temporomandibular disorder, and masticatory myofascial pain syndromes.* Rheumatol Int, 2007. 27(6): p. 541-4.
22. Plesh, O., F. Wolfe, and N. Lane, *The relationship between fibromyalgia and temporomandibular disorders: prevalence and symptom severity.* J Rheumatol, 1996. 23(11): p. 1948-52.
23. Pimentel, M.J., et al., *Features of temporomandibular disorders in fibromyalgia syndrome.* Cranio, 2013. 31(1): p. 40-5.
24. Hedenberg-Magnusson, B., M. Ernberg, and S. Kopp, *Presence of orofacial pain and temporomandibular disorder in fibromyalgia. A study by questionnaire.* Swed Dent J, 1999. 23(5-6): p. 185-92.
25. Gui, M.S., M.J. Pimentel, and C.M. Rizzatti-Barbosa, *Temporomandibular disorders in fibromyalgia syndrome: a short-communication.* Rev Bras Reumatol, 2015. 55(2): p. 189-94.
26. Turp, J.C., et al., *Pain maps from facial pain patients indicate a broad pain geography.* J Dent Res, 1998. 77(6): p. 1465-72.
27. Aggarwal, V.R., et al., *Are reports of mechanical dysfunction in chronic oro-facial pain related to somatisation? A population based study.* Eur J Pain, 2008. 12(4): p. 501-7.
28. Carlson, C.R., et al., *Psychological and physiological parameters of masticatory muscle pain.* Pain, 1998. 76(3): p. 297-307.
29. Cimino, R., et al., *Comparison of clinical and psychologic features of fibromyalgia and masticatory myofascial pain.* J Orofac Pain, 1998. 12(1): p. 35-41.
30. Korszun, A., et al., *The relationship between temporomandibular disorders and stress-associated syndromes.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1998. 86(4): p. 416-20.
31. Van Houdenhove, B. and U.T. Egle, *Fibromyalgia: a stress disorder? Piecing the biopsychosocial puzzle together.* Psychother Psychosom, 2004. 73(5): p. 267-75.
32. Arendt-Nielsen, L., *Central sensitization in humans: assessment and pharmacology.* Handb Exp Pharmacol, 2015. 227: p. 79-102.
33. Siao, P. and D.P. Cros, *Quantitative sensory testing.* Phys Med Rehabil Clin N Am, 2003. 14(2): p. 261-86.
34. Shy, M.E., et al., *Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology.* Neurology, 2003. 60(6): p. 898-904.

35. Backonja, M.M., et al., *Quantitative sensory testing in measurement of neuropathic pain phenomena and other sensory abnormalities.* Clin J Pain, 2009. 25(7): p. 641-7.
36. Geber, C., et al., *Numbness in clinical and experimental pain--a cross-sectional study exploring the mechanisms of reduced tactile function.* Pain, 2008. 139(1): p. 73-81.
37. Klauenberg, S., et al., *Depression and changed pain perception: hints for a central disinhibition mechanism.* Pain, 2008. 140(2): p. 332-43.
38. Uddin, Z. and J.C. MacDermid, *Quantitative Sensory Testing in Chronic Musculoskeletal Pain.* Pain Med, 2016. 17(9): p. 1694-703.
39. Pigg, M., et al., *Reliability of intraoral quantitative sensory testing (QST).* Pain, 2010. 148(2): p. 220-6.
40. Baad-Hansen, L., et al., *Reliability of intra-oral quantitative sensory testing (QST) in patients with atypical odontalgia and healthy controls - a multicentre study.* J Oral Rehabil, 2015. 42(2): p. 127-35.
41. Antonaci, F., T. Sand, and G.A. Lucas, *Pressure algometry in healthy subjects: inter-examiner variability.* Scand J Rehabil Med, 1998. 30(1): p. 3-8.
42. List, T., M. Helkimo, and G. Falk, *Reliability and validity of a pressure threshold meter in recording tenderness in the masseter muscle and the anterior temporalis muscle.* Cranio, 1989. 7(3): p. 223-9.
43. Nothnagel, H., et al., *How stable are quantitative sensory testing measurements over time? Report on 10-week reliability and agreement of results in healthy volunteers.* J Pain Res, 2017. 10: p. 2067-2078.
44. Arendt-Nielsen, L., et al., *Electrophysiological and psychophysical quantification of temporal summation in the human nociceptive system.* Eur J Appl Physiol Occup Physiol, 1994. 68(3): p. 266-73.
45. Skljarevski, V. and N.M. Ramadan, *The nociceptive flexion reflex in humans - review article.* Pain, 2002. 96(1-2): p. 3-8.
46. Sandrini, G., et al., *The lower limb flexion reflex in humans.* Prog Neurobiol, 2005. 77(6): p. 353-95.
47. Chan, C.W. and M. Dallaire, *Subjective pain sensation is linearly correlated with the flexion reflex in man.* Brain Res, 1989. 479(1): p. 145-150.
48. Willer, J.C., *Comparative study of perceived pain and nociceptive flexion reflex in man.* Pain, 1977. 3(1): p. 69-80.
49. Willer, J.C., F. Boureau, and J. Berny, *Nociceptive flexion reflexes elicited by noxious laser radiant heat in man.* Pain, 1979. 7(1): p. 15-20.
50. Banic, B., et al., *Evidence for spinal cord hypersensitivity in chronic pain after whiplash injury and in fibromyalgia.* Pain, 2004. 107(1-2): p. 7-15.
51. Boureau, F., M. Luu, and J.F. Doubrere, *Study of experimental pain measures and nociceptive reflex in chronic pain patients and normal subjects.* Pain, 1991. 44(2): p. 131-138.
52. Desmeules, J.A., et al., *Neurophysiologic evidence for a central sensitization in patients with fibromyalgia.* Arthritis Rheum, 2003. 48(5): p. 1420-9.
53. Guieu, R., G. Serratrice, and J. Pouget, *Counter irritation test in primary fibromyalgia.* Clin Rheumatol, 1994. 13(4): p. 605-10.
54. Curatolo, M., *Diagnosis of altered central pain processing.* Spine (Phila Pa 1976), 2011. 36(25 Suppl): p. S200-4.

55. Micalos, P.S., et al., *Reliability of the nociceptive flexor reflex (RIII) threshold and association with Pain threshold.* Eur J Appl Physiol, 2009. 105(1): p. 55-62.
56. Biurrun Manresa, J.A., et al., *Test-retest reliability of the nociceptive withdrawal reflex and electrical pain thresholds after single and repeated stimulation in patients with chronic low back pain.* Eur J Appl Physiol, 2011. 111(1): p. 83-92.
57. Scaramozzino, P., et al., *Percentile normative values of parameters of electrical pain and reflex thresholds.* Scandinavian Journal of Pain, 2013. 4(2): p. 120-124.
58. Datta, S.C. and M.R. Opp, *Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology.* J Neurosci Methods, 2008. 175(1): p. 119-24.
59. Cheng, J.-K. and R.-R. Ji, *Intracellular Signaling in Primary Sensory Neurons and Persistent Pain.* Neurochemical Research, 2008. 33(10 %@ 1573-6903): p. 1970-1978.
60. Gold, M.S. and G.F. Gebhart, *Nociceptor sensitization in pain pathogenesis.* Nat Med, 2010. 16(11): p. 1248-57.
61. Agostoni, A. and M. Cugno, *[The kinin system: biological mechanisms and clinical implications]*. Recenti Prog Med, 2001. 92(12): p. 764-73.
62. Raz, A. and M. Perouansky, *Central Nervous System Physiology: Neurophysiology*, in *Pharmacology and Physiology for Anesthesia*. 2013. p. 103-122.
63. McMahon, S.B., W.B. Cafferty, and F. Marchand, *Immune and glial cell factors as pain mediators and modulators.* Exp Neurol, 2005. 192(2): p. 444-62.
64. Smith, H.S., *Current therapy in pain*, in *Pathophysiology of Pain*. 2009, Philadelphia : Saunders/Elsevier: Philadelphia. p. 4-8.
65. Ren, K. and R. Dubner, *Interactions between the immune and nervous systems in pain.* Nat Med, 2010. 16(11): p. 1267-76.
66. Watkins, L.R. and S.F. Maier, *Glia: a novel drug discovery target for clinical pain.* Nat Rev Drug Discov, 2003. 2(12): p. 973-85.
67. Tsuda, M., et al., *Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury.* Glia, 2004. 45(1): p. 89-95.
68. Raghavendra, V., F. Tanga, and J.A. DeLeo, *Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy.* J Pharmacol Exp Ther, 2003. 306(2): p. 624-30.
69. Raghavendra, V., M.D. Rutkowski, and J.A. DeLeo, *The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats.* J Neurosci, 2002. 22(22): p. 9980-9.
70. Ledeboer, A., et al., *Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation.* Pain, 2005. 115(1-2): p. 71-83.
71. Grace, P.M., P.E. Rolan, and M.R. Hutchinson, *Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain.* Brain Behav Immun, 2011. 25(7): p. 1322-32.
72. Nicotra, L., et al., *Toll-like receptors in chronic pain.* Exp Neurol, 2012. 234(2): p. 316-29.
73. Rodriguez-Pintó, I., et al., *Fibromyalgia and cytokines.* Immunology Letters, 2014. 161(2): p. 200-203.
74. Uceyler, N., W. Hauser, and C. Sommer, *Systematic review with meta-analysis: cytokines in fibromyalgia syndrome.* BMC Musculoskelet Disord, 2011. 12: p. 245.

75. Wallace, D.J., et al., *Cytokine and chemokine profiles in fibromyalgia, rheumatoid arthritis and systemic lupus erythematosus: a potentially useful tool in differential diagnosis.* Rheumatol Int, 2015. 35(6): p. 991-6.
76. Wallace, D.J., et al., *Cytokines play an aetiopathogenetic role in fibromyalgia: a hypothesis and pilot study.* Rheumatology (Oxford), 2001. 40(7): p. 743-9.
77. Bazzichi, L., et al., *Cytokine patterns in fibromyalgia and their correlation with clinical manifestations.* Clin Exp Rheumatol, 2007. 25(2): p. 225-30.
78. Bazzichi, L., et al., *Antipolymer antibody in Italian fibromyalgic patients.* Arthritis Res Ther, 2007. 9(5): p. R86.
79. Feng, J., et al., *Missense mutations in the MEFV gene are associated with fibromyalgia syndrome and correlate with elevated IL-1beta plasma levels.* PLoS One, 2009. 4(12): p. e8480.
80. Togo, F., et al., *Plasma cytokine fluctuations over time in healthy controls and patients with fibromyalgia.* Exp Biol Med (Maywood), 2009. 234(2): p. 232-40.
81. Zhang, Z., et al., *High plasma levels of MCP-1 and eotaxin provide evidence for an immunological basis of fibromyalgia.* Exp Biol Med (Maywood), 2008. 233(9): p. 1171-80.
82. Caro, X.J. and E.F. Winter, *Evidence of abnormal epidermal nerve fiber density in fibromyalgia: clinical and immunologic implications.* Arthritis Rheumatol, 2014. 66(7): p. 1945-54.
83. Oaklander, A.L., et al., *Objective evidence that small-fiber polyneuropathy underlies some illnesses currently labeled as fibromyalgia.* Pain, 2013. 154: p. 3210-2316.
84. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. 11(5): p. 373-84.
85. Heiman, A., et al., *Toll-like receptors in central nervous system injury and disease: a focus on the spinal cord.* Brain Behav Immun, 2014. 42: p. 232-45.
86. Guo, L.H. and H.J. Schluesener, *The innate immunity of the central nervous system in chronic pain: the role of Toll-like receptors.* Cell Mol Life Sci, 2007. 64(9): p. 1128-36.
87. Mahla, R.S., et al., *Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine Biology.* Front Immunol, 2013. 4: p. 248.
88. Walker, A.K., et al., *Neuroinflammation and comorbidity of pain and depression.* Pharmacol Rev, 2014. 66(1): p. 80-101.
89. Eijkelkamp, N., et al., *GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain.* J Neurosci, 2010. 30(6): p. 2138-49.
90. Cairns, B.E., L. Arendt-Nielsen, and P. Sacerdote, *Perspectives in Pain Research 2014: Neuroinflammation and glial cell activation: The cause of transition from acute to chronic pain?* Scandinavian Journal of Pain, 2015. 6(0): p. 3-6.
91. Willemen, H.L., et al., *Microglial/macrophage GRK2 determines duration of peripheral IL-1beta-induced hyperalgesia: contribution of spinal cord CX3CR1, p38 and IL-1 signaling.* Pain, 2010. 150(3): p. 550-60.
92. Lombardi, M.S., et al., *Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis.* FASEB J, 1999. 13(6): p. 715-25.
93. Vroon, A., et al., *G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis.* J Immunol, 2005. 174(7): p. 4400-6.

94. Fan, J. and A.B. Malik, *Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors.* Nat Med, 2003. 9(3): p. 315-21.
95. Kleibeuker, W., et al., *IL-1 beta signaling is required for mechanical allodynia induced by nerve injury and for the ensuing reduction in spinal cord neuronal GRK2.* Brain Behav Immun, 2008. 22(2): p. 200-8.
96. Kwok, Y.H., et al., *Increased responsiveness of peripheral blood mononuclear cells to in vitro TLR 2, 4 and 7 ligand stimulation in chronic pain patients.* PLoS One, 2012. 7(8): p. e44232.
97. Kwok, Y.H., et al., *TLR 2 and 4 responsiveness from isolated peripheral blood mononuclear cells from rats and humans as potential chronic pain biomarkers.* PLoS One, 2013. 8(10): p. e77799.
98. *The International Classification of Headache Disorders, 3rd edition (beta version).* Cephalalgia, 2013. 33(9): p. 629-808.
99. Smarr, K.L. and A.L. Keefer, *Measures of depression and depressive symptoms: Beck Depression Inventory-II (BDI-II), Center for Epidemiologic Studies Depression Scale (CES-D), Geriatric Depression Scale (GDS), Hospital Anxiety and Depression Scale (HADS), and Patient Health Questionnaire-9 (PHQ-9).* Arthritis Care Res (Hoboken), 2011. 63 Suppl 11: p. S454-66.
100. Spitzer, R.L., et al., *A brief measure for assessing generalized anxiety disorder: the GAD-7.* Arch Intern Med, 2006. 166(10): p. 1092-7.
101. Ohrbach, R., P. Larsson, and T. List, *The jaw functional limitation scale: development, reliability, and validity of 8-item and 20-item versions.* J Orofac Pain, 2008. 22(3): p. 219-30.
102. Campi, L.B., et al., *Painful temporomandibular disorders and central sensitization: implications for management-a pilot study.* Int J Oral Maxillofac Surg, 2017. 46(1): p. 104-110.
103. Alonso-Blanco, C., et al., *Multiple active myofascial trigger points reproduce the overall spontaneous pain pattern in women with fibromyalgia and are related to widespread mechanical hypersensitivity.* Clin J Pain, 2011. 27(5): p. 405-13.
104. Geber, C., et al., *Test-retest and interobserver reliability of quantitative sensory testing according to the protocol of the German Research Network on Neuropathic Pain (DFNS): a multi-centre study.* Pain, 2011. 152(3): p. 548-56.
105. Umeda, M., L.W. Corbin, and K.S. Maluf, *Preliminary investigation of absent nociceptive flexion reflex responses among more symptomatic women with fibromyalgia syndrome.* Rheumatol Int, 2013. 33(9): p. 2365-72.
106. Ang, D.C., et al., *Association of nociceptive responsivity with clinical pain and the moderating effect of depression.* J Pain, 2011. 12(3): p. 384-9.
107. Parise, M., et al., *Clinical use of polysynaptic flexion reflexes in the management of spasticity with intrathecal baclofen.* Electroencephalogr Clin Neurophysiol, 1997. 105(2): p. 141-8.
108. Piletta, P., H.C. Porchet, and P. Dayer, *Central analgesic effect of acetaminophen but not of aspirin.* Clin Pharmacol Ther, 1991. 49(4): p. 350-4.
109. Piletta, P., H.C. Porchet, and P. Dayer, *[Central analgesic effect of paracetamol]*. Schweiz Med Wochenschr, 1990. 120(50): p. 1950-1.

110. Lautenbacher, S., G.B. Rollman, and G.A. McCain, *Multi-method assessment of experimental and clinical pain in patients with fibromyalgia.* Pain, 1994. 59(1): p. 45-53.
111. Vina, J., et al., *Role of mitochondrial oxidative stress to explain the different longevity between genders: protective effect of estrogens.* Free Radic Res, 2006. 40(12): p. 1359-65.
112. Alvergne, A. and V. Hogqvist Tabor, *Is Female Health Cyclical? Evolutionary Perspectives on Menstruation.* Trends Ecol Evol, 2018. 33(6): p. 399-414.
113. Klein, S.L. and C.W. Roberts, *Sex hormones and immunity to infection*. 2010, Heidelberg ; New York: Heidelberg ; New York : Springer Verlag.

Chapter 7. APPENDICES

7.1 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)

Researchers:

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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 10^{th} 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

7.2 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

7.3 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.

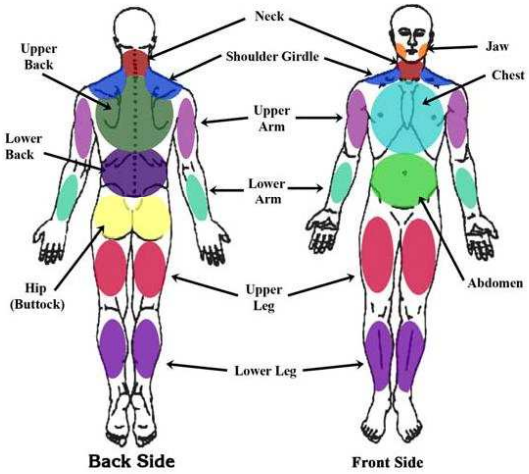
Subject ID: _____

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

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?	☐	☐	☐	☐	☐

7.4 JFLS-20, PHQ-9, GAD-7

Subject ID _____

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

7.5 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)

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

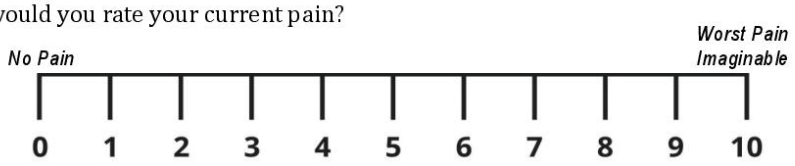
GPCRK2 AS A REGULATOR OF CENTRAL SENSITIZATION IN FIBROMYALGIA AND CHRONIC TEMPOROMANDIBULAR DISORDER

SUBJECT ID _____

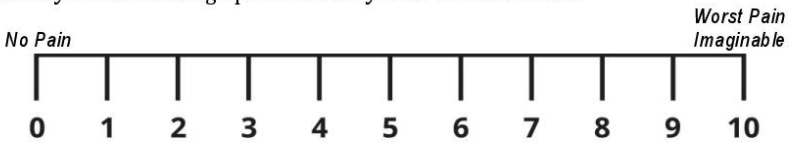
DATE _____

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?

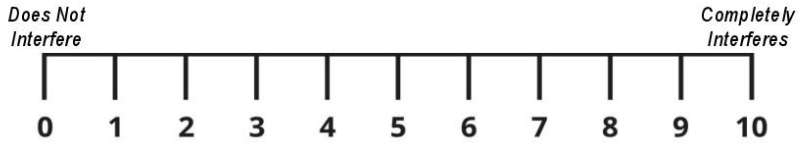


- How would you rate average pain intensity over the last week?

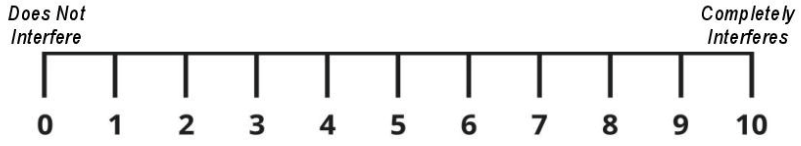


Using the numeric scales below, with 0 representing no interference and 10 representing complete interference, please rate your pain's interference with the following:

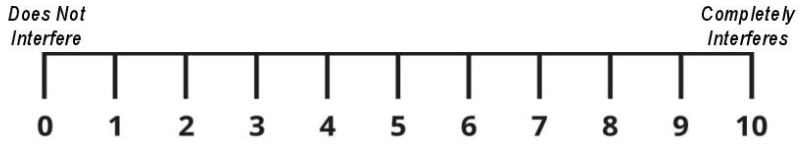
- General activity



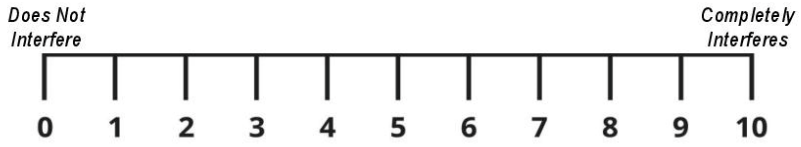
- Enjoyment of life



- Falling asleep



- Staying asleep



7.6 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			

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

SUBJECT ID _____
⊡CONTROL ⊡TMD ⊡ FM
DATE _____

☐ Windup Ratio (0-100)

	Periscapular		Masseter		Dorsum of foot	
Average Ratio						
	1	10	1	10	1	10

☐ Electrical stimulation (mA)

	Sensitivity	1st training	2nd training	3rd training	Actual test
Electrical pain threshold					

	Sensitivity	Manual mode	Randomization mode
Nociceptive Reflex Threshold			___ ___ ___ ___ ___ ___ Average ☐

7.7 BLOOD PROTOCOL

7.7.1 *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.

7.7.2 *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

7.7.3 *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.

7.7.4 *Isolation of PBMCs using cell processing tubes (CPT)*

PBMC Isolation Supplies:

- 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