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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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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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 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 demonstrated 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, IL6, 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 to 13 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 and 1.6. 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 \( \geq 18 \) 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: \( \geq 3 \) 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 10th 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 2nd 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 2nd 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 scapular spine; 3) in the control group, at the center of the pulp of the 2nd 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 cm². 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, 1000 ng/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 °C, 5% CO2 for 24 hr, 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

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

#### 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](image)

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

Abbreviations

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

$x$: 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 IL1\textbeta 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 IL1\textbeta 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
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

<table>
<thead>
<tr>
<th>TLR 2 agonist</th>
<th>10 ng/ml</th>
<th>100 ng/ml</th>
<th>1000 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>SD</td>
<td>Median</td>
</tr>
<tr>
<td>Control</td>
<td>27.0</td>
<td>17.8</td>
<td>27.9</td>
</tr>
<tr>
<td>TMD</td>
<td>15.1</td>
<td>12.8</td>
<td>12.2</td>
</tr>
<tr>
<td>FM</td>
<td>13.9</td>
<td>13.1</td>
<td>10.8</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>496709.4</td>
<td>1</td>
<td>496709.4</td>
<td>44.1</td>
<td>0.000</td>
</tr>
<tr>
<td>Case status</td>
<td>54058.6</td>
<td>2</td>
<td>27029.3</td>
<td>2.4</td>
<td>0.106</td>
</tr>
<tr>
<td>Error</td>
<td>359780.8</td>
<td>32</td>
<td>11243.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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](image)

**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)

<table>
<thead>
<tr>
<th>Measures of CS</th>
<th>Correlation with IL1β level</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT toe (kPa)</td>
<td>-0.167</td>
</tr>
<tr>
<td>PPT masseter (kPa)</td>
<td>0.080</td>
</tr>
<tr>
<td>PPT scapula (kPa)</td>
<td>0.192</td>
</tr>
<tr>
<td>WUR toe</td>
<td>-0.032</td>
</tr>
<tr>
<td>WUR masseter</td>
<td>-0.033</td>
</tr>
<tr>
<td>WUR scapula</td>
<td>-0.025</td>
</tr>
<tr>
<td>Nociceptive flexion reflex threshold (mA)</td>
<td>-0.386</td>
</tr>
<tr>
<td>Electrical pain threshold (mA)</td>
<td>-0.020</td>
</tr>
<tr>
<td>Pain area (No. of pixels)</td>
<td>-0.199</td>
</tr>
</tbody>
</table>

*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 IL1β 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


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:
Michele Curatolo, MD
Lisa Flint
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Principal Investigator & Professor, Anesthesiology
Research Coordinator, Anesthesiology
UW Dental Resident
(206)543-2568
(206)543-7817
(213)503-4663

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

APPROVED

FM or TMD Consent Form
Version 8.5
become painful. You will be instructed to press a button when it reaches that point and
the test will stop. This test is repeated three times (at each location).

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

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

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

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

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

RISKS, STRESS, OR DISCOMFORT

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

Questionnaires

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

Pain sensitivity tests

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

Blood draw

Some people find blood draws uncomfortable. There is a risk of pain, bruise at the point where
the blood is taken, redness or swelling of the vein and infection, and a rare risk of fainting.
Privacy
Although we will make every effort to keep your information confidential, no system for protecting your confidentiality can be completely secure. It is possible that persons might discover that you are in this study, or might obtain information about you.

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

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

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

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

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

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

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

OTHER INFORMATION
You may refuse to participate and you are free to withdraw from this study at any time without penalty or loss of benefits to which you are otherwise entitled.
You will receive $100 for completion of the study. If visit is split into two visits, you will receive $70 for the blood draw and $30 if all procedures except the blood draw is completed. You may be asked for your Social Security number for University of Washington record keeping.

**RESEARCH-RELATED INJURY**

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

<table>
<thead>
<tr>
<th>Printed name of study staff obtaining consent</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
</table>

**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.

<table>
<thead>
<tr>
<th>Printed name of subject</th>
<th>Signature of subject</th>
<th>Date</th>
</tr>
</thead>
</table>

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, Oxecta, 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

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

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 _____.

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Symptom Severity Score (SS score) - Part 2b

Check each of the following OTHER SYMPTOMS that you have experienced over the past week?

- Muscle pain
- Irritable bowel syndrome
- Fatigue/irritability
- 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
- Raynaud’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 AND 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 AND 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 AND 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 would meet the new FM diagnostic criteria.

If your WPI (Part 1) was 5 and your SS score (Parts 2a & b) was 7, then you would NOT 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


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.

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## DC/TMD Examination Form

**Subject ID**

**Examiner**

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

<table>
<thead>
<tr>
<th>Right Pain</th>
<th>Left Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Temporalis</td>
<td>Temporalis</td>
</tr>
<tr>
<td>Other m muscles</td>
<td>Other m muscles</td>
</tr>
<tr>
<td>Non-mast structures</td>
<td>Non-mast structures</td>
</tr>
</tbody>
</table>

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

| None       | None      |
| Temporal   | Temporal  |
| Other      | Other     |

### 2. Incisal Relationships

<table>
<thead>
<tr>
<th>Horizontal Inclined Overjet</th>
<th>Vertical Incisal Overlap</th>
<th>Midline Deviation</th>
<th>Right</th>
<th>Left</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>If negative mm</td>
<td>If negative mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3. Opening Pattern (Supplemental, select all that apply)

<table>
<thead>
<tr>
<th>Vertical Line Deviation</th>
<th>Right</th>
<th>Left</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 4. Opening Movements

#### A. Pain Free Opening

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>mm</td>
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</table>

#### B. Maximum Unassisted Opening

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>mm</td>
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</tbody>
</table>

#### C. Maximum Assisted Opening

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>mm</td>
</tr>
</tbody>
</table>

#### D. Terminated

<table>
<thead>
<tr>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

### 5. Lateral and Protrusive Movements

#### A. Right Lateral

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>mm</td>
</tr>
</tbody>
</table>

#### B. Left Lateral

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
</tr>
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<tbody>
<tr>
<td>mm</td>
<td>mm</td>
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</table>

#### C. Protrusion

<table>
<thead>
<tr>
<th>Right Side</th>
<th>Left Side</th>
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<tbody>
<tr>
<td>mm</td>
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</table>

If negative

<table>
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<tr>
<th>Right</th>
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</tbody>
</table>
# Diagnostic Criteria for Temporomandibular Disorders
## Symptom Questionnaire

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Date</th>
</tr>
</thead>
</table>

### 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?  
   - 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

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. In the last 30 days, have you had any jaw joint noise(s) when you moved or used your jaw?</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>9. Have you ever had your jaw lock or catch, even for a moment, so that it would not open ALL THE WAY?</td>
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<tr>
<td>If you answered NO to Question 9 then skip to Question 13.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Was your jaw lock or catch severe enough to limit your jaw opening and interfere with your ability to eat?</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>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?</td>
<td></td>
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<td></td>
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<tr>
<td>If you answered NO to Question 11 then skip to Question 13.</td>
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</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>12. Is your jaw currently locked or limited so that your jaw will not open ALL THE WAY?</td>
<td></td>
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</tr>
</tbody>
</table>

### CLOSED LOCKING OF THE JAW

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>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?</td>
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<tr>
<td>If you answered NO to Question 13 then you are finished.</td>
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<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>R</th>
<th>L</th>
<th>DNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>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?</td>
<td></td>
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</tbody>
</table>
7.4  JFLS-20, PHQ-9, GAD-7
Jaw Functional Limitation Scale – 20

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

<table>
<thead>
<tr>
<th>No limitation</th>
<th>Severe limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Chew tough food</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>2. Chew hard bread</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>3. Chew chicken (e.g., prepared in oven)</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>4. Chew crackers</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>5. Chew soft food (e.g., macaroni, canned or soft fruits, cooked vegetables, fish)</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>6. Eat soft food requiring no chewing (e.g., mashed potatoes, apple sauce, pudding, pureed food)</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>7. Open wide enough to bite from a whole apple</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>8. Open wide enough to bite into a sandwich</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>9. Open wide enough to talk</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>10. Open wide enough to drink from a cup</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>11. Swallow</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>12. Yawn</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>13. Talk</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>14. Sing</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>15. Putting on a happy face</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>16. Putting on an angry face</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>17. Frown</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>18. Kiss</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>19. Smile</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>20. Laugh</td>
<td>0 1 2 3 4 5 6 7 8 9 10</td>
</tr>
</tbody>
</table>
7.5 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)

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
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

SCREENING AND TESTING RECORD

Assessment Form

BMI:
- Weight: _____ lb → _____ kg
- Height: _____ in → _____ cm → _____ m → _____ m²

Screening

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

- Navigate Pain App:
  - Pain Area: ______

- PHQ-9: ______
  - Function Difficulty: None, Somewhat, Very, Extreme

- GAD-7: ______
  - Function Difficulty: None, Somewhat, Very, Extreme

- 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 _______
Dynamic mechanical alldynia testing (0-100)

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

Intraoral sites-on gingiva/tongue

<table>
<thead>
<tr>
<th>Nerve</th>
<th>Intraoral sites-on gingiva/tongue</th>
<th>Q-tip</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2:ASA</td>
<td>Anterior Sup alveolar n.- buccal #7/#10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2:PSA</td>
<td>Post. Sup Alveolar n.- buccal #3/#10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2:NP</td>
<td>Nasopalatine n.-palatal #7/#10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2:GP</td>
<td>Greater Palatine n.-palatal to #3/#14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3:M</td>
<td>Intraoral Mental n.-gingiva#22/#27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3:LB</td>
<td>Intraoral long buccal n.-gingiva#19/#30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3:LG</td>
<td>Lingual n. - lingual gingiva #21/#28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3:LT</td>
<td>Lingual n. - mid dorsum tongue</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“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)

```
<table>
<thead>
<tr>
<th>Training session (Palm)</th>
<th>Periscapular</th>
<th>Masseter</th>
<th>2nd Toe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual PPT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```
<table>
<thead>
<tr>
<th>Windup Ratio (0-100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periscapular</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electrical stimulation (mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrical pain threshold</td>
</tr>
<tr>
<td>1st training</td>
</tr>
<tr>
<td>Nociceptive Reflex Threshold</td>
</tr>
<tr>
<td>Sensitivity</td>
</tr>
<tr>
<td>Average</td>
</tr>
</tbody>
</table>
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 3<sup>rd</sup> 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 2/3<sup>rd</sup>s 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% CO₂ 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 µL 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 -80°C.

### 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 cat. 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](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

   $$25 \, \mu L \, cells + \, 225 \, \mu L \, Turk's \, Solution: \, \text{Dilution} \, _____ \times \text{Count} \, _____ \times 2500 = \, \text{___________ cells/mL}$$

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 -80°C

13. Log sample location and make sure this patient worksheet is filled out and sample location recorded