Estrogen-Induced Monocytic Response Correlates with Temporomandibular Disorder Pain: A Case Control Study

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Abstract

Temporomandibular disorders (TMD) are a set of conditions characterized by pain and dysfunction in the temporomandibular joint and muscles of mastication. These pain conditions are associated with considerable morbidity, societal costs, and reduced quality of life. The prevalence varies between 4% and 10%, with females at higher risk, and a higher prevalence occurs during reproductive years. The increased prevalence of TMD in females and low prevalence in childhood reinforce that sex hormones, like estrogen, play an important, complex role in the pathophysiology of these disorders. The goal of this study was to determine whether women with TMD exhibit a monocytic hyperinflammatory response compared with control women, and to examine associations of monocytic inflammatory responses with clinical pain. Eighteen women, aged 18 to 35 y, were seen during their follicular menstrual phase. A blood sample was collected, a clinical questionnaire about pain history was administered, and a Research Diagnostic Criteria (RDC) exam was performed. Extracted monocytes were stimulated with the toll-like receptor (TLR)-4 ligand, lipopolysaccharide (LPS), in the presence and absence of estrogen, and the levels of IL6 expression evaluated. Women with TMD showed a systemic hyperinflammatory phenotype, manifested by an increased monocytic release of cytokines after an inflammatory insult, and this was further increased by estrogen. In addition, monocytes from participants who self-reported more pain on the VAS scale produced higher levels of IL6 compared with those from participants who self-reported lower pain sensitivity. These data suggest that an estrogen-induced hyperinflammatory phenotype in women with TMD may at least in part contribute to heightened clinical pain, perhaps via central sensitization.

Keywords: lipopolysaccharide, monocytes, estrogen receptors, inflammation, toll-like receptors, cytokines

Introduction

Temporomandibular disorders (TMD) are a set of conditions characterized by pain and dysfunction in the temporomandibular joint and muscles of mastication, and pain is the main factor leading patients to seek care. The prevalence of TMD varies from 4% to 10%, with females being at higher risk, especially during their reproductive years (Ghurye and McMillan 2015). TMD represents one of several idiopathic pain conditions that show greater prevalence among females and demonstrate substantial comorbidity, including fibromyalgia, migraine and tension-type headaches, and irritable bowel syndrome (Ghurye and McMillan 2015). Improved understanding of the biology responsible for the increased frequency of women with TMD may contribute to better management not only of TMD but also of other pain conditions that disproportionally affect women.

Although multiple gonadal hormones can influence pain processing, estrogens have been the most widely studied and represent one potential factor that may contribute to male–female differences in pain. The highest incidence of TMD occurs in women during their child-bearing years, and there is no difference in the prevalence of TMD between pre-adolescent boys and girls (Ghurye and McMillan 2015). LeResche and colleagues demonstrated that the intensity of TMD pain varies

during the menstrual cycle, with peak pain levels occurring at times of low estrogen and at times of rapid estrogen change (LeResche et al. 2003). During pregnancy, when both estrogen and progesterone are at very high levels, TMD pain substantially declines, and then increases again postpartum, a time of rapid estrogen withdrawal (LeResche et al. 2005). TMD symptoms attenuate after menopause (Kramer and Bellinger 2009), and the use of exogenous hormones, particularly estrogens, is associated with increased incidence and severity of TMD (Wise et al. 2000). Together, these studies strongly implicate estrogens in the pathogenesis of TMD pain.

TMD is characterized by localized pain in the temporomandibular joints and masticatory muscles, which may be related to localized inflammation. However, TMD patients also show

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generalized pain sensitivity, along with a systemic hyperinflammatory phenotype (Slade et al. 2011). This hyperinflammatory phenotype could emerge via the overproduction of cytokines by inflammatory cells in response to sensing through innate immune receptors, such as toll-like receptors (TLRs) (Shaddox et al. 2010). Innate immune receptors are triggered not only by recognition of pathogen-associated molecular patterns but also by endogenous alarmins that are released upon injury and cell death (damage-associated molecular patterns), as well as through changes in the levels of circulating cytokines (Beattie et al. 2002). Evidence from animal studies indicates that the systemic release of cytokines can activate immune-like glial cells within the central nervous system (CNS) (Nicotra et al. 2012), and is central to the initiation and progression of chronic pain (Milligan and Watkins 2009). Glial cells can also become activated in a TLR-dependent manner and release cytokines that can amplify this central sensitization (Milligan and Watkins 2009). Together, these studies strongly implicate TLR-dependent inflammation and pro-inflammatory cytokines in the pathogenesis of pain.

Many cells of the immune system express estrogen receptors (ER), and estrogen is suggested to play a role in regulating their response to stimulation. Some *in vitro* evidence suggests that 17 β -estradiol (E2) induces an anti-inflammatory phenotype in monocyte/macrophage cell lines following lipopolysaccharide (LPS) stimulation, whereas other animal studies report that estrogen induces a pro-inflammatory phenotype in these populations (Villa et al. 2015). The actions of estrogen are regulated via multiple receptors, including ER α , ER β , and the G-protein-coupled receptor, GPR30 (Hazell et al. 2009). The pro-inflammatory effect of estrogen on monocytes and microglial cells is ER α -dependent (Dennison et al. 2012), whereas the anti-inflammatory effect of estrogen seems to be dependent on ER β (Roman-Blas et al. 2010) and GPR30 (Blasko et al. 2009). These studies strongly suggest that estrogen can regulate immune and immune-like cells, and that the effects of estrogen vary based on which ER is activated.

In summary, inflammation and estrogen have independently been implicated in the pathogenesis of TMD pain. In addition, estrogen can regulate the inflammatory profile of immune and immune-like cells in an ER-dependent fashion. Yet, the role of estrogen on immune cell function and the correlations with pain among women with TMD have not been evaluated. Thus, the goals of this study were to 1) evaluate the TLR-responsiveness of immune cells obtained from women with TMD, 2) evaluate the effect of estrogen on the TLR-responsiveness, 3) evaluate the role of estrogen receptors on the inflammatory response, and 4) correlate the TLR-responsiveness with self-reported TMD pain. We hypothesized that monocytes from women with TMD would demonstrate a more robust TLR-responsiveness than those from control participants. We also hypothesized that this increased responsiveness would be influenced by the type and amount of monocytic ER expression and would correlate with higher pain sensitivity in women with TMD.

Materials and Methods

Study Subjects

Twenty-one female participants (11 TMD and 10 controls) were enrolled in the study. Two TMD participants and one control participant were withdrawn from the study during statistical analysis because they were identified as outliers. The number of participants included in the data set was 18 women (9 TMD and 9 controls) (Table 1). All procedures were performed per the ethical guidelines of the University of Florida Institutional Review Board. Potential participants completed telephone screening to determine inclusion criteria: 1) 18 to 35 y, with an absence of 2) ongoing chronic pain other than TMD; 3) uncontrolled hypertension; 4) circulatory disorders; 5) history of cardiac events, metabolic disease, or neuropathy; 6) use of prescription medications including analgesics, tranquilizers, antidepressants, or other centrally acting agents. Furthermore, the participants were confirmed 7) not pregnant; and with 8) no mental health disorders that required hospitalization in the past year. Inclusion for TMD participants: 1) TMD pain of at least 6 mo duration; and 2) at least 10 d of pain over the last 30 d. Participants were scheduled for a clinical session conducted at the University of Florida's Pain Clinical Research Unit during their follicular phase (between days 4 and 9 after the onset of menses). Each participant provided informed consent, a pain self-report questionnaire, a clinical examination using the Research Diagnostic Criteria (RDC) (Dworkin and LeResche 1992), and 5 mL of peripheral blood. Participants who met criteria for both arthralgia and myalgia by the RDC/TMD clinical exam were included in the TMD group. Participants who reported no history of TMD and did not meet criteria for neither arthralgia nor myalgia by the RDC/TMD clinical exam were included in the control group.

Cell Culture

Monocytes were isolated from peripheral blood by Ficoll-Hypaque (Sigma-Aldrich) (Nazarpour et al. 2012). Cells were resuspended in HEPES-buffered, phenol red-free RPMI 1640 (Cellgro; this media lacks E2) supplemented with 10% charcoal dextran-stripped FBS (Hyclone) and 50 mg/mL gentamicin sulfate. Monocytes (5×10^4) were untreated or treated with 500 ng/mL of an ultra-pure TLR4 agonist [*Escherichia coli* LPS] (Invitrogen) in the presence or absence of 10^{-9} M estrogen (Tocris Bioscience) (Cerciat et al. 2010). In some cultures, we used 10 nM of selective antagonists for ER α ([1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride] (MPP) (Tocris Bioscience), ER β ([4-2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl] phenol) (PHTPP) (Tocris Bioscience)] (López-González et al. 2011) and GPR30 ([7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol]) (ICI) (Tocris Bioscience) (Thomas et al. 2005; López-González et al. 2011). RNA and supernatants were harvested at 6 h and 24 h after stimulation, respectively.

Table 1. Cohort Demographic and Pain Characteristics.

		TMD (n = 9)	Control (n = 9)
Cohort demographics and characteristics ^a			
Age, y		26 (7)	25 (7)
Ethnicity, n (%)	Not Hispanic/Latino	7 (78)	8 (89)
	Hispanic/Latino	2 (22)	1 (11)
Race, n (%)	Caucasian	6 (67)	7 (78)
	Black/African American	3 (33)	2 (13)
	Completed high school	11	0
Schooling, %	Some college	33	33
	College	45	45
	Postgraduate	11	22
Marital Status	Never married	67	78
	Divorced	11	0
	Living as married	11	11
	Married	11	11
Pain characteristics			
Duration of TMD		7.75 (5.175)	n/a
Worst pain in last 6 mo ^b		7.33 (2.062)	n/a
Average pain in last 6 mo ^b		5.11 (2.369)	n/a
Pain interference in the last 6 mo ^b		3.89 (3.00)	n/a
Pain interference with daily activity ^b		8.56 (2.00)	n/a
Pain interference with work activity ^b		2.00 (1.00)	n/a
Pain interference with social life ^b		2.00 (1.00)	n/a

TMD, temporomandibular disorder; n/a, not applicable.

^aData presented are the mean (SEM), except where indicated.

^bValues on a scale of 0 to 10.

Flow Cytometry

For ER expression, monocytes were washed in phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS) and probed for ER α and ER β . Manufacturer-recommended isotype controls were used as negative controls for all tested antibodies and staining was performed at a 1:100 dilution. Data were acquired using an Acurri Flow Cytometer (BD Biosciences) and analyzed using FlowJo data analysis software (FlowJo LLC).

ELISA

ELISA (BD Biosciences) was used to test for IL6 expression within 100 μ l of supernatant, as per the manufacturer's protocol. Data were acquired on a spectrophotometer (BioTek), analyzed using a standard curve, and are presented as pg/mL.

Real-time PCR

Total RNA from monocytic cells was harvested using an RNAeasy extraction Kit (Qiagen) and reverse transcribed using reverse transcription master mix [5 $\times$ buffer, 1 mM DTTs, 2.5 mM dNTPs, SuperScript III (Invitrogen), and oligonucleotides]. Primers specific for IL6 (Integrated DNA Technologies) were used for qPCR reactions. Data were collected and analyzed using CFX Connect and CFX Manager (Bio-Rad Laboratories) as per the *ddCT* algorithm and using 18S as a reference gene.

Statistics

Statistical analyses were performed using SPSS version 23.0 (IBM SPSS Statistics for Windows; IBM Corp). All continuous variables were assessed for normality using the Shapiro–Wilk test. Data are presented as either mean and SEM or box-and-whisker plot (displaying the first and third quartiles at the ends of the box, the median, and the maximum and minimum at the ends of the whiskers). Data were compared using a Student's *t* tests for groups of 2, and one-way ANOVA with Bonferroni's multiple corrections for groups of 3 or more. A median split on self-reported pain was determined to dichotomize participants into groups that self-reported high vs. low pain, and a Student's *t* test was performed. Pearson's correlation coefficient was used to correlate self-reported pain with IL6 within the TMD cohort. Significance was determined at $P < 0.05$.

Results

Elevated Unstimulated Monocytic IL6 Expression in TMD

IL6 is a multifunctional cytokine induced following experimental pain (Zhang and An 2007; Wei et al. 2013), and associated with several clinical pain conditions (Starkweather 2010; Wei et al. 2013). We first assessed for the differential expression of IL6 in the 24-h conditioned supernatants of unstimulated monocytic cells from participants with TMD and control participants using ELISA. We found that unstimulated monocytes

Figure 1. Elevated IL6 in temporomandibular disorder (TMD)-associated monocytes at rest. Monocytes (1×10^4) isolated from the peripheral blood of participants with ($n = 9$) and without ($n = 9$) TMD were (A, B) unstimulated or (C–E) stimulated with 500 ng/mL of ultra-pure LPS from *Escherichia coli*. The 24-h conditioned supernatants were assessed for IL6 expression by ELISA. (A, C) Data are presented as pg/ml. Bars represent mean and SEM. Black, TMD; white, control (CON). * $P = 0.0286$; ^ $P = 0.002$; independent Student's t test. (B, D) Data are presented as fold change over control: (B) 8.2 (1.03) and (D) 12.6 (3.38). (E) Data are presented as fold change of mRNA over unstimulated control. Bars represent mean and SEM. Black, TMD; gray, control. * $P = 0.0241$; Student's t test.

from the TMD cohort expressed 8 times the amount of IL6 than unstimulated monocytes cells from the control cohort ($P = 0.0286$; Fig. 1A, B).

Enhanced TLR4-induced Expression of IL6 in TMD

Although innate sensing of tissue destruction by monocytes is predominantly associated with infection, it is also an important aspect of wound healing, including that observed in the remodeling of the temporomandibular joint (TMJ) (Karki and Igwe 2013). Innate sensing can also induce IL6 expression (O'Shea and Plenge 2012). To evaluate the innate sensing of monocytes from TMD and control participants, isolated monocytes were stimulated with a TLR4-specific inducer of IL6, ultra-pure LPS. To compensate for the exaggerated expression of IL6 in the absence of stimulation observed in the TMD cohort, the unstimulated values were subtracted from the TLR4-induced expression levels. TLR4-stimulated monocytic cells from TMD participants expressed 12-fold more IL6 than TLR4-stimulated monocytic cells from control participants ($P =$

0.0020; Fig. 1C, D). This increase in protein expression was verified at the mRNA level, with TLR4-stimulated monocytes from TMD participants expressed more IL6 mRNA than TLR4-stimulated control monocytes ($P = 0.0241$; Fig. 1E).

Estrogen Exacerbates the Enhanced TLR4-induced Expression of IL6 in TMD

Most immune cells express ERs (ER α , ER β , or the membrane-bound G-protein-coupled ER) to varying degrees and can thus respond to estrogen (Raghavendra et al. 2002). These receptors have demonstrated the capacity to regulate a spectrum of immune functions, including cytokine production. We next evaluated the effect of estrogen on the TLR4-induced monocytic production of IL6. Estrogen enhanced the TLR4-induced expression of IL6 by monocytes from both TMD and control participants ($P = 0.04$ and $P = 0.02$ respectively; Fig. 2A). However, when estrogen was present during TLR4-stimulation, monocytic cells from TMD participants demonstrated a 14-fold greater induction of IL6 release compared with that from control counterparts ($P = 0.002$; Fig. 2B). This increase in protein expression was verified at the mRNA level, with estrogen enhancing the mRNA expression of IL6 following TLR4-stimulation of monocytes from both cohorts (Fig. 2C). Importantly, the estrogen-induced augmentation of the response was significantly higher in monocytes from participants with TMD ($P < 0.05$; Fig. 2C).

The type of ER can regulate the effect of estrogen in inflammatory conditions; thus, we next assessed the expression levels of ER α and ER β in resting monocytes isolated from the peripheral blood of TMD and control participants. Although there was no significant difference in the expression of ER α (Fig. 3A), ER β was expressed at significantly lower levels in samples from TMD-derived monocytes (Fig. 3A). To determine the contribution of each ER subtype to the augmentation of TLR4-stimulation by estrogen, we used neutralizing antibodies against ER α , ER β and/or GPR30 in the estrogen- and TLR4-induced IL6 responses observed. Neutralization of ER β (E2+MPP) or GPR30 (E2+ICI) was most effective in suppressing the estrogen-induced TLR4-responses in TMD- and non-TMD derived monocytes (Fig. 3B). Most importantly, when only ER α was inhibited in TMD-derived monocytes, the estrogen-induced TLR4-response was still equivalent to the estrogen-induced TLR4-response observed in monocytes from controls (Fig. 3B).

TLR4-induced Monocytic IL6 Release Correlates with Self-reported Orofacial Pain Level

Several studies have reported that excessive production of IL6 in the synovial fluid might contribute to TMD synovitis and arthralgia (Vernal et al. 2008). Thus, the severity of self-reported clinical TMD pain using the VAS scale was correlated with the monocytic TLR4 responsiveness within the TMD cohort. When the TMD participants were stratified into those with low self-reported pain (VAS <4.5) vs. those with high self-reported pain (VAS >4.5), a significant difference in the

IL6 production by monocytes was observed (Fig. 4A). Higher levels of IL6 were induced following stimulation with TLR4 ligand alone and in combination with estrogen in monocytes from participants with higher VAS scores (Fig. 4A), and a positive correlation was observed between VAS scores and TLR4-induced monocytic IL6 expression in the absence (Fig. 4B) and presence (Fig. 4C) of estrogen.

Discussion

Inflammation can modulate pain responses both peripherally and centrally, yet the role of inflammation in TMD-associated pain remains unclear. Peripheral inflammation can play a role in the pathogenesis of some TMD (Kristensen et al. 2011) after trauma and overloading of the joints and muscles. These events could lead to inflammatory insults, such as muscle damage, synovitis, internal derangement, and osteoarthritis, producing peripheral sensitization in the trigeminal region. Therefore, inflammation may represent an initiating event contributing to the development of TMD-pain. Previous studies have correlated increased pro-inflammatory cytokines in the TMJ synovial fluid with TMJ pain. Recent evidence also has demonstrated increased levels of circulating pro-inflammatory cytokines in TMD (Slade et al. 2011), suggesting the presence of low-grade, systemic inflammation in TMD. Induction of systemic inflammation in humans produces a generalized enhancement in pain sensitivity (Karshikoff et al. 2016), and sustained low-grade systemic inflammation can promote a shift in microglia toward a pro-inflammatory state, resulting in the release of cytokines into the CNS and thus neural inflammation (Barrientos et al. 2010). In recent years, a new concept has emerged suggesting that distinct subsets of monocytes and macrophages exist in both the periphery and in target tissues, respectively, which differ in their ability to produce cytokines and growth factors in response to specific stimuli. Our data suggest that women with TMD have a systemic hyperinflammatory phenotype, manifested by increased monocytic release of IL6 after stimulation with a TLR4 ligand. Moreover, among the TMD participants, the level of IL6 released was positively correlated with their self-reported pain. These data support the hypothesis that women with TMD can present with a hyperresponsive inflammatory system and this hyperinflammatory phenotype can induce central sensitization, contributing to heightened clinical pain.

Several studies citing the prevalence of TMD in females and the low prevalence in childhood have demonstrated that estrogen has a complex role in both inflammatory and pain responses. (LeResche 1997). For instance, microglial cells of the CNS, which express ER, can become activated in response to inflammatory stimuli, releasing cytokines and other mediators that are pronociceptive and amplify the pain response centrally (Manson 2010). Some evidence suggests that estrogen can induce an

Figure 2. Estrogen enhances TMD-associated monocytic TLR4-hyperresponsiveness. Monocytes (1×10^4) isolated from the peripheral blood of participants with ($n = 9$) and without ($n = 9$) TMD, were stimulated with 500 ng/mL of ultra-pure LPS from *Escherichia coli* in the presence or absence of 10^{-9} M 17β -estradiol (E2). Conditioned supernatants were then examined for IL6 expression by ELISA and qPCR after 24 h and 6 h, respectively. **(A)** Data are presented as pg/mL. Bars represent mean and SEM. Black, TMD; gray, control; cross-hatched, E2. * $P = 0.04$ (TMD); $P = 0.02$ (CON) LPS vs. E2; Student's *t* test. **(B)** Data are presented as fold change over control. Mean fold change is 14.2 (4.31). (A, B) Bars represent mean and SEM. Black, TMD; gray, control; cross-hatched, E2. **(C)** Data are presented as fold change of mRNA for treated over unstimulated using box-and-whisker plot. Black, TMD; gray, control; cross-hatched, E2. * $P < 0.0001$, LPS vs. E2; $^{\wedge}P < 0.0001$ TMD vs. CON. One-way ANOVA with Bonferroni's multiple corrections.

anti-inflammatory phenotype in monocyte or macrophage cell lines following their activation (Vegeto et al. 2004), whereas other animal studies have reported an estrogen-induced pro-inflammatory phenotype in the same cell populations (Calippe et al. 2010). Our data demonstrate that estrogen significantly enhances the inflammatory response of monocytes derived from both TMD and control participants. Most importantly, this more pronounced effect also correlates with self-reported pain.

The role of estrogen in inflammation and pain are complex, and this has led to contradictory findings in the literature, including those presented in this study. It is important to note that the concentration of estrogen determines its effect on inflammation and on pain responses. Specifically, at low concentrations (such as that observed during the follicular phase, early preovulatory phase, postmenopause, and following ovariectomy), estrogen induces a pro-inflammatory process. Importantly, this coincides with times of peak pain levels in TMD (LeResche et al. 2003). Conversely, at high concentrations (such as those observed during ovulation and pregnancy), estrogen inhibits inflammatory processes, and this corresponds with conditions under which TMD-associated pain has been reported to subside. The average concentration of serum estrogen levels during the mid-follicular phase ranges from 27 to 123 pg/mL, whereas estrogen levels during the periovulatory phase range from 96 to 436 pg/mL. Thus, it is imperative to know the stage of the menstrual cycle during which data or samples are collected, as well as the relative concentration of estrogen tested in the *in vitro* assays to interpret and compare study findings. Our evaluation of pain ratings and our sample collection were performed when participants were in the early follicular phase of the menstrual cycle, and we evaluated the effect of estrogen at a concentration that is observed during the transition from the follicular phase to the periovulatory phase (~ 272.38 pg/mL). Thus, overall, our study evaluated the effect of low estrogen concentrations on inflammation and TMD pain.

In addition to estrogen, ER expression also fluctuates throughout the menstrual cycle. In human, ER expression is

Figure 3. Estrogen receptor (ER) use in TMD-associated monocytic TLR4-hyperresponsiveness. Monocytes (1×10^4) isolated from the peripheral blood of participants with ($n = 9$) and without ($n = 9$) TMD were (A) unstimulated or (B) stimulated with 500 ng/mL of ultra-pure LPS from *Escherichia coli* in the presence and absence of 10^{-9} M 17 β -estradiol (E2) or 10 nM of inhibitors to ER α (MPP), ER β (PTHP) and ER α + ER β inhibitor, GPR30 agonist (ICI) (see Methods for inhibitors). (A) The relative surface expression of ER α and ER β was determined by flow cytometry. Data are presented as the mean fluorescence intensity, which is directly proportional to the number of receptors expressed on the cell surface of a given cell. Data are presented as box-and-whisker plots. Black, TMD; gray, control; * $P < 0.05$. TMD vs. CON for ER β . One-way ANOVA with Bonferroni's multiple corrections. (B) Data are presented as fold change over expression following TLR4-stimulation using box-and-whisker plots. Black, TMD; gray, control. * $P < 0.05$. TMD vs. CON E2. $^{\Delta}P < 0.05$ E2 vs. all other stimulations within an experimental group. One-way ANOVA with Bonferroni's multiple corrections.

higher in the follicular phase, peaks at mid-cycle, and declines in the late luteal phase (Jansen et al. 2001). In addition, the pro-inflammatory effect of estrogen appears to be ER α -dependent (Douin-Echinard et al. 2008), whereas the anti-inflammatory

effect of estrogen seems to be dependent on ER β (Roman-Blas et al. 2010) and GPR30 (Blasko et al. 2009). Thus, distinguishing between the relative expression of each ER may be more important than the overall ER expression. Animal studies have demonstrated that ER β expression declines in the early follicular phase, providing an additional layer of regulation to the effect that low estrogen may have on inducing inflammatory responses (Jansen et al. 2001). Here, we demonstrated that monocytes from TMD participants expressed significantly lower levels of only ER β , even though all cells were collected at similar phases of the menstrual cycle. In addition, whereas neutralization of ER α , ER β , and GPR30 abrogated the enhanced inflammation mediated by estrogen, neutralization of ER β was not as effective on monocytes collected from the TMD cohort. Together, these data shed light on the ER mechanisms that may contribute to the more robust estrogen-mediated augmentation of inflammation seen in monocytes from TMD participants.

We do acknowledge the limitations of this study. Despite demonstrating statistical significance, the sample size evaluated was small. In addition, we did not evaluate the effect of high physiological concentrations of estrogen or other phases of the menstrual cycle. Also, we recognize that inflammation is a well-recognized contributor to arthrogenous TMD, whereas its role in myogenous pain is less well understood. Due to the small sample size in this pilot study, we are unable to compare these diagnostic subgroups. Finally, we only evaluated one aspect of inflammation and only one mediator of that response. These issues are currently being addressed by our research program.

In conclusion, this preliminary study is the first to demonstrate the presence of a TLR4-monocytic hyperresponsive phenotype in women with TMD. In addition, we demonstrate that estrogen concentrations, such as those observed during the follicular phase of the menstrual cycle, exacerbate hyperresponsiveness, which is influenced by the type and amount of monocytic ER expression. Most importantly, our data suggest that this hyperresponsiveness also correlates with self-reported pain in women with TMD. Our findings pave the way for the identification of biological pathways within which to intervene to improve not only TMD-associated pain in women but also other painful conditions that disproportionately affect women.

Author Contributions

M.C. Ribeiro-Dasilva, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; R.B. Fillingim, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; S.M. Wallet, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure 4. TMD-associated monocytic TLR4-hyper-responsiveness correlates with self-reported pain. IL6 levels from monocytic cultures stimulated with 500 ng/mL of ultra-pure LPS from *Escherichia coli* in the presence and absence of 10^{-9} M 17 β -estradiol (E2) were log transformed to determine relative expression levels. **(A)** VAS scores from TMD participants were stratified as low self-reported pain (VAS <4.5; black circles) and high self-reported pain (VAS >4.5; white circles). Data are presented as mean and SEM of relative expression of IL6 as well as relative expression for each participant (each dot represents a single participant). * $P < 0.0137$. Student's t test. **(B)** Within the TMD cohort, VAS scores and relative expression levels of IL6 following TLR4 stimulation were correlated using Pearson's correlation ($P = 0.0328$). **(C)** Within the TMD cohort, VAS scores and relative expression levels of IL6 following TLR4 and E2 stimulation were correlated using Pearson's correlation ($P = 0.0338$).