Title

Effect of antidepressant treatment on plasma levels of neuroinflammation-associated molecules in patients with somatic symptom disorder with predominant pain around the orofacial region

Running Head

Plasma cytokines in chronic orofacial pain

Keywords

somatic symptom disorder with predominant pain; burning mouth syndrome; atypical odontalgia; neuroinflammation; cytokines; chemokines

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Abstract

OBJECTIVE. Burning mouth syndrome (BMS) and atypical odontalgia (AO) are examples of somatic symptom disorders with predominant pain around the orofacial region. Neuroinflammation is thought to play a certain role in the mechanisms, but few studies have been conducted about the involvement of neuroinflammation. We aimed to better understand the role of neuroinflammation in the pathophysiology and treatment of BMS/AO.

METHODS. Plasma levels of 28 neuroinflammation-related molecules were determined in 44 controls and 48 BMS/AO patients both pre- and 12 weeks post-treatment with duloxetine.

RESULTS. Baseline plasma levels of interleukin (IL)-1β ($P < 0.0001$), IL-1 receptor antagonist ($P < 0.001$), IL-6 ($P < 0.0001$), macrophage inflammatory protein-1β ($P < 0.0001$) and platelet-derived growth factor-bb ($P = 0.04$) were significantly higher in patients than in controls. Plasma levels of granulocyte macrophage colony stimulating factor were significantly higher in patients than in controls ($P < 0.001$) and decreased with treatment ($P = 0.009$). Plasma levels of eotaxin, monocyte chemoattractant protein-1 and vascular endothelial growth factor decreased significantly with treatment ($P < 0.001$, $P = 0.022$, and $P = 0.029$, respectively).

CONCLUSIONS. Some molecules may be involved in the pathophysiology and/or treatment response of somatic symptom disorders with predominant pain around the orofacial region.

1. Introduction

The impact of chronic pain extends far beyond the symptoms of pain itself. Chronic pain drastically affects the lives of patients and their significant others, and has major effects on societies and economies (Edwards, Dworkin, Sullivan, Turk, & Wasan, 2016; Turk, Wilson, & Cahana, 2011). Complex interactions between biological factors, cognitive factors, emotional factors, and previous history can contribute to chronic pain. However, the detailed mechanisms underlying the pathophysiology of these conditions remain unclear. Although various treatments are available for non-cancer pain, such as antidepressants and pregabalin/gabapentin (Turk, et al., 2011), their effectiveness is fairly limited.

Pain can become chronic in various regions of the body, including the orofacial region. Representative diseases involving chronic pain around the orofacial region include burning mouth syndrome (BMS) and atypical odontalgia (AO). BMS is defined as "an intraoral burning or dysesthetic sensation, recurring daily for more than 2 h/day over more than 3 months, without clinically evident causative lesions" (Headache Classification Subcommittee of the International Headache Society, 2018). AO is thought to be a subform of persistent idiopathic facial pain (PIFP). PIFP is described as "persistent facial and/or oral pain, with varying presentation but recurring daily for more than 2 h/day over more than 3 months, in the absence of clinical neurological deficit". Additionally, "the term AO has been used to describe a continuous pain in one or more teeth or in a tooth socket after extraction, in the absence of any usual dental cause" (Headache Classification Subcommittee of the International Headache Society, 2018). The primary psychiatric diagnosis applied to both conditions is somatic symptom disorder with predominant pain, according to the diagnostic and statistical manual of mental disorders, 5th edition (DSM-5) (American Psychiatric Association, 2013). These diseases are idiopathic and tend to be treatment resistant. To make matters worse, persistent pain is associated with a hotbed of psychiatric symptoms such as depression and anxiety (McMillan, et al., 2016; Melis, et al., 2003). The prevalence of BMS is reportedly 0.7-15% and disproportionately affects postmenopausal women (McMillan, et al., 2016). AO develops in 3-6% of patients who undergo root canal treatments and most commonly affects females 40 years of age or older (Melis, et al., 2003). Thus, BMS/AO are relatively common conditions that are often treatment resistant, and new and effective therapeutics are urgently needed. We are grappling with this difficult problem, and previously reported that the antidepressant, duloxetine, had some effect on chronic pain around the orofacial

region (Nagashima, et al., 2012) but that this effect was not related to its plasma concentration (Kobayashi, et al., 2017).

Recently, it has been suggested that neuroinflammation may play a certain role in the pathogenesis of chronic pain around the orofacial region (Melis, et al., 2003; Yilmaz, et al., 2007; Baad-Hansen, 2008). In neuroinflammation, neuronal-glial interactions mediated by proinflammatory cytokines and chemokines lead to plastic changes of the peripheral and central nervous systems. This phenomenon may underlie the development and persistence of chronic pain (Ji, Berta, & Nedergaard, 2013; Benarroch, 2010). Neuroinflammation may play a very significant role in chronic orofacial pain. Several studies have evaluated levels of proinflammatory and anti-inflammatory cytokines in the saliva and blood of patients with BMS (Simcić, et al., 2006; Boras, Brailo, Lukac, Kordić, & Blazić-Potocki, 2006; Suh, Kim, & Kho, 2009; Pekiner, Demirel, Gümrü, & Ozbayrak, 2008; Chen, Xia, Lin, Zhou, & Li, 2007). These studies have yielded conflicting results regarding the levels of neuroinflammatory mediators in saliva. Likewise, the number of studies reporting associations between blood cytokine levels and BMS is quite small. Two studies have suggested an association between interleukin (IL)-1β polymorphisms and the pathogenesis of BMS (Kim, Kim, Chang, Kim, & Kho, 2017; Guimarães, et al., 2006). Chemokines are small chemotactic cytokines and are involved in neuroinflammation at different anatomical locations, where they contribute to chronic pain processing (Abbadie, et al., 2009). Nevertheless, few studies have examined the role of chemokines in chronic orofacial pain. To the best of our knowledge, only two studies have evaluated levels of IL-8 in saliva and plasma of patients with BMS (Suh, et al., 2009; Barry, O'Halloran, McKenna, McCreary, & Downer, 2018), and only one study reported changes in levels of neuroinflammation-related molecules induced by treatment (Pezelj-Ribarić, et al., 2013). In the latter study, levels of IL-6 and tumor necrosis factor (TNF)-α were measured before and after low-level laser therapy. No studies have measured levels of neuroinflammation-related molecules in patients with AO. However, it seems likely that neuroinflammation plays a common role in the pathogenesis of both AO and BMS.

If neuroinflammation was ~~were~~ involved in BMS/AO, one presumes that inflammation would be alleviated by treatment. However, the results of previous studies have not been sufficient to reach this conclusion. We aimed to examine more broadly whether neuroinflammation was associated with somatic symptom disorder with predominant pain around the orofacial region, and whether levels of neuroinflammation-related molecules were affected by treatment. IL-1β, IL-2, IL-6,

IL-10, TNF-α, and interferon (IFN)-γ are major proinflammatory/anti-inflammatory cytokines which are thought to be involved in neuropathic pain. There is abundant evidence that proinflammatory cytokines, particularly IL-1β, IL-6, and TNF-α, are involved in processing of pathological pain (Zhang & An, 2007; Moalem & Tracey, 2006). Potential involvement of IL-2 and IFN-γ has also been suggested (Davies, Hayes, & Dekaban, 2007; Uçeyler, Eberle, Rolke, Birklein, & Sommer, 2007; Uçeyler, Rogausch, Toyka, & Sommer, 2007; Vikman, Siddall, & Duggan, 2005; Vikman, Duggan, & Siddall, 2007). IL-10 is a potent anti-inflammatory cytokine, repressing the secretion of inflammatory cytokines by activated macrophages (Zhang & An, 2007; Strle, et al., 2001). Plasma levels of these inflammatory mediators were measured with various accuracy in prior studies of patients with BMS. Therefore, we measured them in our study using chemiluminescent enzyme immunoassays (CLEIAs) and enzyme-linked immunosorbent assays (ELISAs) as precisely as possible. We exhaustively surveyed plasma levels of other molecules in BMS/AO patients, including chemokines, using a multiplex magnetic bead-based immunoassay. To our best knowledge, ours is also the first study to examine the effect of duloxetine, an antidepressant, on plasma levels of neuroinflammation-associated molecules.

2. Subjects & Methods

2.1. Participants

The subjects enrolled in this prospective study were 48 individuals, aged 20 years or older, who visited the Liaison Outpatient Clinic of Aichi Gakuin University Dental Hospital between April 2010 and February 2014. These individuals were diagnosed with BMS and/or AO by dentists and somatic symptom disorder with predominant pain by trained psychiatrists according to the DSM-5 based on clinical interview, and our diagnosis was validated by the examination every 2 weeks. Our clinic diagnosed patients using DSM-IV-TR criteria until 2013, and since that time we have used the DSM-5. We re-classified the diagnoses of patients with pain disorder who had been seen and diagnosed during or prior to 2013 using the DSM-5 criteria. All patient diagnoses were consistent with somatic symptom disorder with predominant pain. The 48 participants were treated with duloxetine for 12 weeks (20 mg/day for the first weeks and 40 mg/day thereafter). The duloxetine dose was decreased to 20 mg/day in patients experiencing tolerability issues. Only alprazolam (up to 1.2 mg/day) and brotizolam (up to 0.5 mg/day) were used in combination with duloxetine for management of anxiety or insomnia. Out of ~~Of~~ 164 individuals with BMS/AO, 116 patients were

excluded based on the criteria described below, because of the absence of informed consent, or because of duloxetine discontinuation. Exclusion criteria were as follows: (i) diagnosis of major depressive disorder, defined using the DSM-5 criteria, at initial consultation; (ii) history of schizophrenia or other psychotic disorders, or obvious current psychotic symptoms; (iii) clinically overt dementia; (iv) any serious physical diseases; (v) previous use of duloxetine; and (vi) use of any psychotropic agent within 2 weeks of participation in the study. For exclusion criterion (vi), enrollment was permitted if patients discontinued drug use for 2 weeks. During this period, the patients were treated with only alprazolam (up to 1.2 mg/day) and brotizolam (up to 0.5 mg/day) as necessary to relieve discontinuation symptoms. These inclusion and exclusion criteria were the same as those used by Nagashima et al. (Nagashima, et al., 2012; Kobayashi, et al., 2017). 44 healthy control individuals were made up of volunteers recruited from the school of dentistry, Aichi Gakuin University and Nagoya University Hospital. Their ages and genders were broadly matched with the BMS/AO patients and they had no orofacial pain or psychiatric disorders.

2.2. Ethics statement

The study was conducted in accordance with the principles laid out in the Declaration of Helsinki, and was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine (No. 2004-0234-2) and the Ethical Committee of the School of Dentistry, Aichi-Gakuin University (No. 372). Every effort was made to protect patients' confidentiality and personal information. All participants provided written informed consent.

2.3. Clinical Assessment

At the time of inclusion and every 2 weeks until 12-week follow-up, the intensity of pain was measured using a visual analogue scale (VAS). Patients were instructed to bisect a 10-cm line, drawn from 0 (no pain) to 100 (extreme pain), at a point appropriate to quantify their pain or discomfort. 6 out of 48 BMS/AO patients had missing VAS data at baseline and/or at 12-week follow-up and were excluded from further analysis.

2.4. Collection of plasma and analysis

Blood samples were collected from healthy controls at the time of inclusion and from BMS/AO patients at inclusion and at 12-week follow-up. Plasma was prepared from whole blood by centrifugation at 3,500×g for 5 min, transferred to sterile 2-mL

microcentrifuge tubes using a pipette, and stored at −30° C until further analysis.

A CLEIA was used to quantitate IL-6 (Fujirebio, Shinjuku-ku, Tokyo, Japan) and five separate ELISAs were used to quantitate IL-1β (R&D Systems, Minneapolis, Minnesota, USA), IL-2 (R&D Systems), IL-10 (Thermo Fisher Scientific, Minato-ku, Tokyo, Japan), TNF-α (Thermo), and IFN-γ (Thermo). Chemokines and other neuroinflammation-related molecules were measured using the Bioplex Pro Human Cytokine 27-Plex multiplex assay (Bio-rad, Hercules, California, USA). The molecules quantitated by this multiplex assay included IL-1 receptor antagonist (IL-1ra), IL-4, IL-5, IL-7, IL-9, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma-induced protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, platelet-derived growth factor-bb (PDGF-bb), regulated on activation, normal T cell expressed and secreted (RANTES), and vascular endothelial growth factor (VEGF). Concentrations of high sensitivity C-reactive protein (hsCRP), high levels of which are indicative of nonspecific inflammation, were measured with a JCA-BM6050 instrument (JEOL, Akishima, Tokyo, Japan). The plasma volume used for ELISA, CLEIA, Bioplex, and JCA-BM6050 instrument was 50 ul, 50 ul, 25 ul, and 250 ul respectively. All tests were performed according to the manufacturers' instructions for each kit, and each assay was performed in duplicate using appropriately diluted plasma samples. The final data represented the average of duplicate measurements. The lower detection limits of each molecule are as follows: 0.1 pg/mL for IL-1β, IL-2, and IL-6; 3.03 pg/mL for IL-1ra; 0.26 pg/mL for IL-4; 0.82 pg/mL for IL-5; 0.01 pg/mL for IL-7; 2.36 pg/mL for IL-8; 0.15 pg/mL for IL-9; 1.0 pg/mL for IL-10; 2.16 pg/mL for IL-12; 0.38 pg/mL for IL-13; 1.4 pg/mL for IL-15; 0.78 pg/mL for IL-17; 1.7 pg/mL for TNF-α; 4.0 pg/mL for IFN-γ; 1.628 pg/mL for eotaxin; 0.492 pg/mL for bFGF; 2.15 pg/mL for G-CSF; 0.2 pg/mL for GM-CSF; 1.41 pg/mL for IP-10; 1.674 pg/mL for MCP-1; 0.03 pg/mL for MIP-1α; 0.388 pg/mL for MIP-1β; 0.87 pg/mL for PDGF-bb; 0.81 pg/mL for RANTES; 2.077 pg/mL for VEGF; and 0.001 mg/dL for hsCRP. Values below the lower limit of detection were recorded as the threshold value for further analyses.

2.5. Statistical analyses

The Mann-Whitney U-test was used to compare baseline plasma levels of molecules between BMS/AO patients and healthy controls at the time of inclusion. The Wilcoxon Signed Rank test was used to compare plasma levels of molecules and VAS values within patient subgroups over time. Spearman's rank correlation coefficients were

calculated to investigate relationships between VAS values and plasma concentrations of molecules whose levels significantly changed before and after duloxetine treatment. A power analysis was performed using G* power 3.1.9.3. Statistical significance was assumed for $P < 0.05$.

3. Results

3.1. Participant characteristics

A total of 92 subjects were recruited in this study (48 BMS/AO patients and 44 healthy controls). The demographic data of participants, dental diagnosis, disease durations, and final doses of duloxetine are presented in Table 1.

3.2. VAS values

VAS values were significantly lower ($P < 0.0001$) in BMS/AO patients at 12-week follow-up (median: 29.5, IQR: 11.3-47.8) compared with the baseline (median: 54.5, IQR: 39.8-79.8). They are shown in Figure 1. The ratio of patients with a VAS score improvement rating of 30% or greater and of 50% or greater was 30/42 and 19/42, respectively.

3.3. Plasma levels of neuroinflammation-related molecules

Plasma levels of molecules of interest were measured in controls and BMS/AO patients at the time of inclusion as well as in patients at 12-week follow-up. Of the molecules measured using the magnetic bead-based multiplex assay, only data for IL-1ra, IL-7, IL-9, IL-17, eotaxin, MCP-1, MIP-1α, MIP-1β, bFGF, G-CSF, GM-CSF, PDGF-bb, and VEGF were used, as levels of other molecules were outside the range of the calibration curve (data not shown). IL-2, IL-10, TNF-α, and IFN-γ were measured using ELISA and were completely undetectable in all participants (data not shown).

Plasma levels of the remaining molecules (IL-1β, IL-1ra, IL-6, IL-7, IL-9, IL-17, eotaxin, MCP-1, MIP-1α, MIP-1β, bFGF, G-CSF, GM-CSF, PDGF-bb, VEGF, and hsCRP) were evaluated and recorded in Table 2 and 3. Molecules showing significant differences between controls and patients at baseline and/or between patients at baseline and at 12-week follow-up are shown in Figures 2, 3, and 4 (IL-1β, IL-1ra, IL-6, MCP-1, MIP-1β, eotaxin, GM-CSF, PDGF-bb, and VEGF).

Plasma levels of IL-1β ($P < 0.0001$), IL-1ra ($P < 0.001$), IL-6 ($P < 0.0001$), MIP-1β ($P < 0.0001$), and PDGF-bb ($P = 0.04$) were significantly higher in BMS/AO patients at baseline compared with healthy controls. No statistical significances

in plasma levels of these five molecules were observed between patients at baseline and at 12-week follow-up. Plasma levels of eotaxin ($P < 0.001$), MCP-1 ($P = 0.022$), and VEGF ($P = 0.029$) did not differ between controls and patients at baseline but were lower in patients at 12-week follow-up after treatment than at baseline. Plasma GM-CSF concentrations were significantly higher at baseline in BMS/AO patients than in controls ($P < 0.001$) and decreased in patients at 12-week follow-up compared with baseline ($P = 0.009$).

The correlations between levels of the four substances showing significant changes associated with duloxetine treatment and VAS values were as follows: eotaxin ($r = 0.19$, $P = 0.24$), MCP-1 ($r = 0.39$, $P = 0.02$), GM-CSF ($r = 0.53$, $P < 0.001$), and VEGF ($r = 0.22$, $P = 0.15$).

3.4. Power analysis

This study had a power of 0.07 to detect a small effect, 0.28 to detect a medium effect, and 0.64 to detect a large effect in the Mann-Whitney U-test; had a power of 0.10 to detect a small effect, 051 to detect a medium effect, and 0.91 to detect a large effect in the Wilcoxon Signed Rank test. In the Spearman’s rank correlation coefficients, this study had a power of 0.25 for eotaxin, 0.80 for MCP-1, 0.98 for GM-CSF, and 0.33 for VEGF.

4. Discussion

To our knowledge, this is the first study to report levels of a large panel neuroinflammation-related molecules (not limited to proinflammatory/anti-inflammatory cytokines) in patients with chronic orofacial pain, and to analyze changes in the concentrations of these substances induced by treatment with antidepressants. Our results demonstrated that plasma levels of some molecules differed between controls and BMS/AO patients at baseline and that levels of other molecules in patients differed before and after treatment with duloxetine.

Chronic orofacial pain is a neuropathic pain in which neuroinflammation plays an essential role, and prior studies have reported differences in levels of proinflammatory and anti-inflammatory cytokines in BMS patients compared with controls. For example, saliva IL-2 and IL-6 levels were significantly elevated in BMS patients (Simcić, et al., 2006). By contrast, another study reported no significant differences in salivary levels of IL-6 and TNF-α in BMS patients (Boras, et al., 2006). It has been pointed out that levels of these substances may be influenced

by the degree of blood contamination (Suh, et al., 2009). Peniker et al. (2008) measured serum levels of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ in BMS patients and observed decreased IL-2 and TNF-α levels in these patients. Chen et al. (2007) found that serum IL-6 levels decreased in BMS patients and were negatively correlated with the magnitude of pain. IL-6 can exert both detrimental or beneficial effects on the nervous system; these effects may depend on the timing and level of IL-6 expression (Wyss-Coray & Mucke, 2002; Biber, et al., 2008). Barry et al. measured plasma levels of IL-8 and showed that these levels were higher in BMS patients compared with controls; moreover, levels increased according to the intensity of pain and depression (Barry, et al., 2018). However, this was only a pilot study involving a small number of individuals. As mentioned above, measurements of neuroinflammation-associated molecules in saliva have yielded inconsistent results (Simcić, et al., 2006; Boras, et al., 2006; Suh, et al., 2009), and studies using blood samples have sporadically suggested that some cytokines might exert some influence on BMS pathophysiology (Pekiner, et al., 2008; Chen, et al., 2007; Barry, et al., 2018). Two genomic studies showed that IL-1β polymorphisms are associated with the pathology of BMS (Kim, et al., 2017; Guimarães, et al., 2006). Taken together, the data suggest that neuroinflammation is involved in chronic orofacial pain.

Only one previous study by Pezelj-Ribarić et al. (2013) reported changes in levels of neuroinflammation-related molecules induced by treatment. In this study, low-level laser therapy was administered to BMS patients, and levels of IL-6 and TNF-α in saliva were decreased after treatment. However, blood contamination in saliva samples can affect these measurements (Suh, et al., 2009), and thus changes in levels of these molecules might not reflect improvement of pain. This could explain why VAS was not significantly decreased after low-level laser therapy in this study.

Although limited numbers of proinflammatory/anti-inflammatory cytokines have been examined by some studies of BMS described above, neuroinflammation consists of complex molecular cascades in which many mediators are involved. Our study surveyed more molecules than previous studies and compared levels before and after duloxetine treatment. Furthermore, in our study, duloxetine treatment improved pain symptoms, unlike the results of Pezelj-Ribarić et al. (2013). The degree of improvement we observed was similar to that reported in a study in which patients with pain disorders around the orofacial region were treated with the same antidepressant (Nagashima, et al., 2012). Our results strongly suggested the involvement of neuroinflammation in BMS/AO. The number of enrolled patients in our study was equal to that of the study by Chen et al. (2007), and was larger than most previous studies. The diagnosis and

evaluation of patients were shared between dentists and psychiatrists from the time of enrollment until 12-week follow-up, and we targeted a homogeneous patient group.

Among the molecules we measured here, plasma levels of IL-1β, IL-1ra, IL-6, MCP-1, MIP-1β, eotaxin, GM-CSF, PDGF-bb, and VEGF differed significantly between controls and BMS/AO patients at baseline and/or between patients at inclusion and at 12-week follow-up after treatment. Plasma levels of IL-1β, IL-1ra, IL-6, MIP-1β, PDGF-bb, and GM-CSF were higher in BMS/AO patients at inclusion than in controls, and thus these molecules may contribute to development and persistence of somatic symptom disorder with predominant pain around the orofacial region. It is well known that IL-1β and IL-6 play roles in pain persistence as parts of neuroinflammatory networks. MIP-1β is upregulated under inflammatory conditions and has been implicated in the pathogenesis of several diseases (Menten, Wuyts, & Van Damme, 2002). MIP-1β mediator is upregulated in injured nerves and participates in neuropathic pain (Saika, Kiguchi, Kobayashi, Fukazawa, & Kishioka, 2012). PDGF is involved in several cellular responses such as differentiation and gene expression (Andrae, Gallini, & Betsholtz, 2008; Heldin, Ostman, & Rönnstrand, 1998). PDGF is expressed in dorsal horn neurons and is involved in neuropathic pain through microglial stimulation (Narita, et al., 2005; Masuda, Tsuda, Tozaki-Saitoh, & Inoue, 2009). IL-1ra interferes with IL-1β binding to its receptor and suppresses the action of IL-1β (Braddock & Quinn, 2004). Enhancement of nociceptive neuron responses induced by IL-1β is abrogated by pretreatment with IL-1ra (Oka, Aou, & Hori, 1994). In our study, high level of IL-1ra in BMS/AO patients may have been responses against the development and maintenance of chronic pain.

Our study demonstrated that plasma levels of GM-CSF in BMS/AO patients were higher than in controls and decreased after duloxetine treatment. GM-CSF can promote peripheral sensitization as a pain mediator (Schweizerhof, et al., 2009). GM-CSF is induced by IL-1 in many cell types and may be an important downstream mediator of IL-1 biology (Ren & Torres, 2009). Taking our results and strong correlation with VAS together, we speculate that IL-1 is involved in both the pathophysiology of BMS/AO and in response to treatment. Inhibition of IL-1 may lead to improvement in pain.

In BMS/AO patients, plasma levels of eotaxin, MCP-1, and VEGF decreased significantly after duloxetine treatment. While eotaxin levels vary among reports on different diseases (Chalan, et al., 2016; Sharifabadi, et al., 2014), several reports have suggested that eotaxin levels are increased in individuals with neuropathic pain (Bäckryd, et al., 2017; Makker, et al., 2017). MCP-1 can contribute to development of chronic pain through central sensitization and microglial

activation (Thacker, et al., 2009). VEGF promotes angiogenesis, increases blood vessel permeability, and is responsible for different functions of the nervous system. VEGF is involved in the pathogenesis of neuropathic pain through actions on dorsal root ganglion neurons (Lin, et al., 2010). From these findings, we speculate that all of these substances may participate in chronic pain in some way, and our results demonstrate that changes in the levels of these molecules might be associated with response to treatment. Some of these changes may be due to indirect effects of duloxetine, because direct action of this antidepressant has not been demonstrated (Kremer, M., et al. 2016).

Plasma levels of hsCRP showed no significant differences between controls and BMS/AO patients, which may suggest that these patients do not show the characteristics of classical inflammation.

It is suggested that gender difference may affect the immune system (Imahara, et al., 2005). Although sample size was small, we analyzed the levels of all molecules measured in this study using the Mann-Whitney U-test, and found that there was not significant difference in the levels between male and female in healthy control, patients at baseline, and patients at 12-week follow-up respectively. Therefore, it is considered that the influence on the concentration due to gender difference is small.

We did not store plasma samples in -80° C but in -30° C freezer, which was not standard procedure. However, we compared healthy control samples stored under the same condition with those of patient group, and also used samples that were not repeatedly frozen and thawed. Therefore, it seems to be reliable data.

There are several limitations to the present study. First, it is difficult for the negative results to be interpreted definitely into several statistical analyses due to small sample size. Our sample size is larger than those in past studies; however, a still larger sample would provide more robust results. Second, we did not consider the possibility of placebo effects. To better observe the effects of duloxetine treatment on levels of neuroinflammation-related molecules, we would require a placebo group. Third, we did not divide the BMS/AO patient group into drug responders and non-responders. Molecules whose levels were higher in patients than in controls and did not decrease with antidepressant treatment might play a role in treatment resistance. To validate this hypothesis, future large-scale studies will be needed. Fourth, we did not divide the patient group according to comorbidity with major depression. Depression is major comorbidity of chronic pain and could be associated with neuroinflammation. Plasma levels of neuroinflammation-related

molecules may change according to the presence or absence of depression in patients. Fifth, the biological mechanisms underlying BMS/AO have not been elucidated. When these become clearer, the meaning of the differences observed in plasma levels of inflammatory mediators may become clearer.

In conclusion, our results provide further support to the notion that neuroinflammation is involved in chronic orofacial pain and that neuroinflammation-related molecules are associated with response to treatment. Multiplexed assays allow for screening of multiple analytes using small volumes of plasma samples, and specific findings from this study can be followed with targeted enzyme immunoassays. In particular, GM-CSF may be a promising biomarker in BMS/AO, and more detailed investigations of this molecule are required. We hope that our findings contribute to clarifying the underlying mechanisms of BMS/AO and improve the treatment of somatic symptom disorder with predominant pain around orofacial region.

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Figure captions

Figure 1. Visual analogue scale (VAS) values in burning mouth syndrome/atypical odontalgia patients at baseline and at 12‐week follow‐up (n = 42). The median VAS value at baseline was 54.5 (IQR: 39.8-79.8) and at 12‐week follow‐up was 29.5 (IQR: 11.3-47.8). These results demonstrated a significant decrease in VAS associated with duloxetine treatment ($p < .0001$).

Figure 2. Molecules with higher baseline plasma levels in burning mouth syndrome/ atypical odontalgia patients than in healthy controls. Four, two, and three outliers are not shown in panels b, c, and e, respectively.

Figure 3. Molecules whose levels were lower in burning mouth syndrome/atypical odontalgia patients at 12‐week follow‐up than at baseline.

Figure 4. Molecules whose levels were higher in burning mouth syndrome/atypical odontalgia (BMS)/atypical odontalgia (AO) patients at baseline than in healthy controls and whose levels were lower in BMS/AO patients at 12‐week follow‐up compared with the baseline in these individuals.

Table 1. Plasma levels of neuroinflammation-related molecules in controls and BMS/AO patients at baseline

	plasma levels[a], pg/ml		
	controls (n = 44)	at baseline (n = 48)	*P*-value
IL-1β	0.10 [0.10-0.10]	0.20 [0.10-0.25]	< 0.0001
IL-1ra	3.03 [3.03-30.52]	25.27 [13.09-44.00]	< 0.001
IL-6	1.50 [0.80-2.58]	3.05 [2.30-4.53]	< 0.0001
IL-7	0.010 [0.010-2.98]	0.86 [0.010-2.08]	0.58
IL-9	3.87 [2.60-4.82]	2.57 [0.15-6.32]	0.091
IL-17	18.21 [10.32-32.68]	18.61[9.5-24.75]	0.38
Eotaxin	344.45 [256.17-480.58]	345.33 [274.56-447.00]	0.46
MCP-1	41.98 [29.15-63.39]	45.97 [29.33-75.45]	0.47
MIP-1α	1.16 [1.16-1.44]	1.34 [0.68-2.19]	0.27
MIP-1β	47.98 [41.47-55.35]	72.86 [50.22-103.24]	< 0.0001
FGF-basic	5.70 [4.03-7.75]	5.63 [4.08-8.65]	0.49
G-CSF	10.28 [6.71-16.18]	15.14 [8.60-22.77]	0.14
GM-CSF	2.13 [0.97-3.16]	4.05 [1.75-6.10]	< 0.001
PDGF-bb	3129.13 [1.07-8852.83]	4828.99 [3020.45-7674.36]	0.04
VEGF	32.49 [21.30-47.32]	37.38 [29.29-60.71]	0.075
hsCRP	0.035 [0.016-0.10][b]	0.027 [0.016-0.061][b]	0.36

[a] Values are medians [interquartile range].

[b] mg/dl

IL: interleukin, MCP: monocyte chemoattractant protein, MIP: macrophage inflammatory protein, FGF: fibroblast growth factor, G-CSF: granulocyte colony stimulating factor, GM-CSF: granulocyte macrophage colony stimulating factor, PDGF: platelet-derived growth factor, VEGF: vascular endothelial growth factor, hsCRP: high sensitivity C-reactive protein.

Table 2. Plasma levels of neuroinflammation-related molecules in BMS/AO patients at baseline and at 12-week follow-up

	plasma levels, pg/ml		
	at baseline (n = 48)	**at 12 wks f/u (n = 48)**	***P*-value**
IL-1β	0.2 [0.1-0.25]	0.23 [0.1-0.29]	0.17
IL-1ra	25.27 [13.09-44.00]	20.39 [12.99-35.05]	0.055
IL-6	3.05 [2.30-4.53]	3.30 [2.48-4.55]	0.68
IL-7	0.86 [0.010-2.08]	1.34 [0.010-2.57]	0.79
IL-9	2.57 [0.15-6.32]	2.85 [0.15-7.19]	0.91
IL-17	18.61[9.5-24.75]	13.72 [8.95-26.32]	0.43
Eotaxin	345.33 [274.56-447.00]	313.63 [220.38-396.94]	< 0.001
MCP-1	45.97 [29.33-75.45]	44.80 [29.31-66.44]	0.022
MIP-1α	1.34 [0.68-2.19]	1.04 [0.52-1.67]	0.1
MIP-1β	72.86 [50.22-103.24]	72.39 [46.61-94.44]	0.13
FGF-basic	5.63 [4.08-8.65]	5.6 [3.53-10.14]	0.49
G-CSF	15.14 [8.60-22.77]	13.94 [9.30-17.13]	0.14
GM-CSF	4.05 [1.75-6.10]	3.19 [1.43-5.42]	0.009
PDGF-bb	4828.99 [3020.45-7674.36]	5677.93 [2166.56-9191.13]	0.66
VEGF	37.38 [29.29-60.71]	31.84 [20.56-61.80]	0.029
hsCRP	0.035 [0.016-0.10]	0.030 [0.018-0.056]	0.22

[a] Values are medians [interquartile range].

[b] mg/dl

IL: interleukin, MCP: monocyte chemoattractant protein, MIP: macrophage inflammatory protein, FGF: fibroblast growth factor, G-CSF: granulocyte colony stimulating factor, GM-CSF: granulocyte macrophage colony stimulating factor, PDGF: platelet-derived growth factor, VEGF: vascular endothelial growth factor, hsCRP: high sensitivity C-reactive protein.