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Neuropeptides CRH, SP, HK-1 and inflammatory cytokines IL-6, TNF are increased in serum of patients with Fibromyalgia Syndrome implicating mast cells

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Running Title Page

Running Title: Neuroimmune markers in fibromyalgia

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Abbreviations:
Blood-brain-barrier (BBB); cerebrospinal fluid (CSF); chronic fatigue syndrome (CFS); corticotropin releasing hormone (CRH); enzyme-immunosorbent assay (ELISA); Fibromyalgia Syndrome (FMS); hemokinin-1 (HK-1); interstitial cystitis/bladder pain syndrome (IC/BPS); irritable bowel syndrome (IBS); mast cells (MCs); myogenic temporomandibular disorder (TMD); neurotensin (NT); Peripheral Blood Mononuclear cells (PBMCs); phosphate-buffered saline (PBS); post-traumatic stress disorder (PTSD); Pain Visual Analog Scale (PVAS); substance P (SP); tumor necrosis factor (TNF); vascular endothelial growth factor (VEGF).

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Abstract

Fibromyalgia Syndrome (FMS) is a chronic, idiopathic condition of widespread musculoskeletal pain, affecting more women than men. Even though clinical studies have provided evidence of altered central pain pathways, the lack of definitive pathogenesis or reliable objective markers has hampered development of effective treatments. Here we report that the neuropeptides corticotropin-releasing hormone (CRH), substance P (SP) and its structurally related Hemokinin-1 (HK-1) were significantly ($p=0.026$, $p<0.0001$ and $p=0.002$, respectively) elevated (0.82±0.57 ng/mL, 0.39±0.18 ng/mL and 7.98±3.12 ng/mL, respectively) in the serum of patients with FMS as compared to healthy controls (0.49±0.26 ng/mL, 0.12±0.1 ng/mL and 5.71±1.08 ng/mL, respectively). Moreover, SP and HK-1 levels were positively correlated (Pearson's $r=0.45$, $p=0.002$) in FMS. The serum concentrations of the inflammatory cytokines IL-6 and TNF were also significantly ($p=0.029$ and $p=0.006$, respectively) higher (2.97±2.35 pg/mL and 0.92±0.31 pg/mL, respectively) in the FMS group as compared to healthy subjects (1.79±0.62 pg/mL and 0.69±0.16 pg/mL, respectively). In contrast, serum IL-31 and IL-33 levels were significantly lower ($p=0.0001$ and $p=0.044$, respectively) in the FMS patients (849.5±1005 pg/mL and 923.2±1284 pg/mL, respectively) in comparison to healthy controls (1281±806.4 pg/mL and 3149±4073 pg/mL, respectively). FMS serum levels of neurotensin were not different from controls. We had previously shown that CRH and SP stimulate IL-6 and TNF release from mast cells (MCs). Our current results indicate that neuropeptides could stimulate MCs to secrete inflammatory cytokines that contribute importantly to the symptoms of FMS. Treatment directed at preventing the secretion or antagonizing these elevated neuroimmune markers, both centrally and peripherally, may prove to be useful in the management of FMS.

Introduction

Fibromyalgia Syndrome (FMS) is a chronic, medical condition characterized by widespread musculoskeletal pain, soft tissue tenderness, sleep dysfunction, stiffness, fatigue, and cognitive dysfunction (Clauw, et al., 2011; Schmidt-Wilcke and Clauw, 2011; Clauw, 2014; Theoharides, et al., 2015b) FMS is estimated to affect 2–8% of the adult population and is considered to be the most common cause of generalized, musculoskeletal pain in women between the ages of 20 and 55 years. (Branco, et al., 2010) Some investigators believe that FMS belongs to a family of overlapping conditions such as chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS), functional dyspepsia, myogenic temporomandibular disorder (TMD), tension headache, myofacial pain syndrome, restless leg syndrome, interstitial cystitis/bladder pain syndrome (IC/BPS), post-traumatic stress disorder (PTSD) and Gulf War syndrome. (Yunus, 2007; Theoharides, 2013) FMS may be triggered by Lyme disease, but in that case, antibiotics do not appear to be effective. (Dinerman and Steere, 1992) It should be pointed out that the pathogenesis of most of these conditions is controversial, so as new information accumulates, they will likely appear to be less similar to each other than is currently perceived. Even though many patients with FMS can meet diagnostic criteria for CFS, there are distinct differences between these two groups. (Abbi and Natelson, 2013)

Diagnostic criteria for FMS have developed as a work-in-progress over the last ten years, (McBeth and Mulvey, 2012;Wolfe and Walitt, 2013) but reliable diagnostic or prognostic laboratory markers would still be welcome additions. Recent research regarding the etiology of FMS has focused on central sensitization, neuroinflammation, allergens, infectious agents, irritants, chemical exposures, or physiologic stress. (Russell, 1998; Arnold, 2010;Russell, et al., 1994) The mediators of inflammation would include neuropeptides, cytokines, growth factors

and neurotransmitters. Indeed, some neurochemical mediators have been found to be abnormal in patients with FMS. (Russell and Larson, 2009; Ceko, et al., 2012)

Mast cells (MCs) are involved in allergic conditions, (Theoharides, et al., 2015f) but can also be viewed as mediators of systemic inflammation. (Theoharides, et al., 2010a; Galli, et al., 2008b) It has been suggested that MCs may be involved in FMS, (Lucas, et al., 2006; Pollack, 2014) as well as other comorbid conditions. (Theoharides, 2013) The numbers of MCs were significantly increased in the papillary dermis of FMS patients. (Blanco, et al., 2010) Moreover, chronic urticaria, which involves MCs, has been identified in some FMS patients. (Torresani, et al., 2009) However, the numbers of MCs may not be as important as the degree of their activation or the specific mediators that they secrete.(Theoharides, et al., 2012)

Activated MCs release numerous vasoactive, neurosensitizing and pro-inflammatory mediators (Theoharides, et al., 2015f) that could contribute to FMS symptoms. (Theoharides, et al., 2010a) In particular, MCs can release certain mediators selectively (Theoharides, et al., 2007) as well as IL-6 without degranulation. (Kandere-Grzybowska, et al., 2003) MCs are located perivascularly in close proximity to neurons both in the skin (Paus, et al., 2006) and in the diencephalon. (Rozniecki, et al., 1999; Dimitriadou, et al., 1997) Increased levels of the neuropeptides corticotropin-releasing hormone (CRH) (Theoharides, et al., 1995) and substance P (SP) may be secreted in response to physiological or psychological stress and could then stimulate MCs to secrete TNF.(Theoharides, et al., 2010b)

Here we report that the neuropeptides CRH, SP, and its structurally related Hemokinin-1 (HK-1), as well as the cytokines IL-6 and TNF, are significantly elevated in the serum of patients with FMS when compared to healthy controls.

Methods

Blood was obtained from patients (all female 28-64 years of age) with FMS. Their demographic characteristics are shown in Table 1. About 70% of these patients were medically referred to one of the authors (IJR) for clinical evaluation and as potential candidates for an ongoing research study about FMS. The source of the medical referrals was an academic secondary care orthopedic clinic of the Bexar County Health Care System, affiliated with the University of Texas Health Science Center at San Antonio, to which they had been referred by their primary care physicians because of hand pain. At screening by the orthopedic physician assistants, who had been previously-trained in the FMS examination by one of the authors [IJR], those patients were found to meet clinical criteria for FMS and not to have convincing evidence for a compressive neuropathy [e.g., carpal tunnel syndrome, CTS]. The remaining 30% were self-referred because of a physician- or presumptive self-diagnosis of FMS. All of the participants met 1990 American College of Rheumatology Research Classification Criteria for a diagnosis of FMS and had a pain severity of at least 40 mm, using the Pain Visual Analog Scale (1-100 mm). Potential subjects were excluded if they had a comorbid rheumatic disease, severe osteoarthritis in weight-bearing joints, an unstable or untreated endocrinopathy, any clinically significant abnormality in screening clinical laboratory tests, a severe debilitating organ failure (including heart disease, renal failure, hepatic failure), systemic cancer in the prior 6 months, uncontrolled systemic hypertension, dementia, aphasia, or other deficits of cognition or speech/language function, a history of drug or alcohol dependence, having taken long-acting opioids in the prior three months, lactating or pregnant women, women of child-bearing potential without a mechanical/barrier or oral anti-conceptive treatment, having fainted within the last six months, having received an investigational drug or device within 30 days prior to starting the

study. The clinical protocol of this research study was approved by the University of Texas Health Science Center in San Antonio Institutional Review Board (IRB#901-5003-203). Patients signed informed consent to participate in the study before any research-related procedures were initiated. Serum was separated from other blood components using standard SST tube methodology. The samples were number coded and no personal identifiers or protected health information accompanied the samples. Within 30 minutes of collection, the serum was aliquoted and frozen at -70^{0}C until analyzed.

Serum was also obtained from healthy subjects (5 male and 15 female, 25-65 years of age) who had no history of musculoskeletal pain or any evidence of inflammation. The normal controls were not related to any of the FMS patient group and were recruited from the Boston area or purchased from BBI Solutions (Cardiff, U.K.). Serum samples were labeled only with a code number, the age and gender of the subjects. All control blood samples were processed immediately after phlebotomy and the serum was stored in -80^{o}C until it was used for analysis.

The numbers of patients included in the various measurements varied because the amounts of serum available were not consistent for all samples. The levels of SP were measured first and as subsequent measurements were made, the amounts of serum remaining decreased progressively until quantities were not sufficient for some additional tests. This was true for both the FMS and normal control serum samples.

Extraction of serum peptides

The extraction of serum peptides was performed using a SEP-COLUMN containing 200 mg of C18 (Cat. No. RK-SEPCOL-1), Buffer A (Cat. No. RK-BA-1) and Buffer B (Cat. No. RK-BB-1) (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). A combination of a centrifugal concentrator (Savant Speedvac SVC 100H) and a lyophilizer (Edwards K4 Modulyo Freeze Dryer, West Sussex,

England) was used for drying the samples after extraction. First, Speedvac was used to dry the samples for approximately 15 minutes in order to remove the organic layer. The remaining sample was snap-frozen in liquid nitrogen and freeze-dried overnight using a lyophilizer. The dried extracts were then reconstituted with 1x assay buffer and phosphate-buffered saline (PBS) for measurement of CRH and neurotensin (NT) in human serum samples.

Assessment of markers levels in serum

Human serum CRH, SP and NT levels were measured using commercially available enzyme-immunosorbent assay (ELISA) kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) according to the manufacturers' protocol. HK-1 levels were measured using commercially available ELISA kits from Peninsula Laboratories International, Inc. (San Carlos, CA, USA). Human serum IL-31, IL-6, IL-33 and TNF levels were determined using commercially available ELISA kits from R&D Systems (Minneapolis, MN, USA).

Statistical Analysis

All of the data were validated and inspected for outliers. The results are presented as scattergrams with symbols representing individual data points and the horizontal lines representing the mean for each group. Normality of distribution was checked with the Shapiro-Wilk's test. Comparisons between the FMS group and the healthy controls were performed using the non-parametric Mann-Whitney U-test or the unpaired t-test. Pearson correlation analysis was performed between SP and HK-1 serum levels in FMS patients. A result was considered significant at a P value <0.05. The analysis was performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA).

Results

The demographic characteristics of the subjects included in this study are presented in Table 1. The FMS patients consisted of 84 females with a mean age of 47.9±11.2 years. Ninety percent were Caucasian (35% European Caucasian, 55% Hispanic Caucasian). The average educational achievement was 13.6 years. Forty percent were gainfully employed. The healthy control group consisted of 15 females and 5 males mean age of 38.2±10.6 years. One hundred percent were Caucasian. The important differences between FMS and healthy control groups were related to diagnosis of a painful multimodal medical condition in the FMS group, but not present in the healthy control group.

Serum levels of the neuropeptides CRH and SP were significantly ($p=0.026$ and $p<0.0001$, respectively) elevated (0.82 ± 0.57 ng/mL and 0.39 ± 0.18 ng/mL, respectively) in patients with FMS as compared to healthy controls (0.49 ± 0.26 ng/mL and 0.12 ± 0.1 ng/mL, respectively) (Fig. 1A, B). However, there was no correlation between CRH and SP serum levels.

The serum levels of HK-1 were increased ($p=0.002$) in FMS patients (7.98 ± 3.12 ng/mL) as compared to normal controls (5.71 ± 1.08 ng/mL) (Fig.2A). Moreover, there was a significant correlation between serum levels of HK-1 and SP ($r=0.45$, $p=0.002$), but not CRH (Fig. 2B).

There was no statistical difference in serum levels of NT between FMS patients and healthy controls (data not shown).

The mean serum levels of the cytokines IL-6 and TNF were significantly ($p=0.029$ and $p=0.006$, respectively) increased (2.97 ± 2.35 pg/mL and 0.92 ± 0.31 pg/mL, respectively) in patients with FMS as compared to healthy controls (1.79 ± 0.62 pg/mL and 0.69 ± 0.16 pg/mL, respectively) (Fig. 3A, B).

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Serum IL-31 and IL-33 levels were significantly ($p=0.0001$ and $p=0.044$, respectively) lower (923.2 ± 1284 pg/mL and 849.5 ± 1005 pg/mL, respectively) in patients with FMS when compared with the healthy controls (3149 ± 4073 pg/mL and 1281 ± 806.4 pg/mL, respectively) (Fig. 4A, B).

Discussion

The demographic characteristics of the FMS and control subjects were comparable (Table 1), but it is important to point out that the FMS group was characterized only by painful multimodal symptoms not present in the healthy control group. Hence, the differences in the biomarkers reported here are clinically significant. In particular, our study shows that the neuropeptides CRH, SP and the SP-related HK-1 are significantly elevated in the serum of FMS patients in comparison to controls. This is the first time to our knowledge that CRH and HK-1 are shown to be increased in the serum of FMS patients.

The neuroimmune findings reported here fit in a "unified" model proposed earlier, but based on CSF measurements. (Russell and Larson, 2009) One study showed that CRH was elevated in the cerebrospinal fluid (CSF) of FMS patients and was associated with pain, but not fatigue symptoms. (McLean, et al., 2006) The increased serum CRH reported in the current study may indicate some level of physical or psychological stress that may exacerbate FMS symptoms. (Geenen, et al., 2002; Fischer, et al., 2015; Fischer, et al., 2015) CRH is typically secreted from the hypothalamus under the influence of stress and activates the HPA axis, but has also been shown to induce inflammation. (Chrousos, 1995) CRH-positive nerve endings have been localized in the median eminence of the hypothalamus, where MCs are most plentiful. (Rozniecki, et al., 1999) CRH can also be released from MCs (Kempuraj, et al., 2004), other immune cells (Karalis, et al., 1997) and skin cells. (Donelan, et al., 2006b) In fact, human MCs express mRNA and protein for CRHR-1, activation of which induces selective release of vascular endothelial growth factor (VEGF) (Cao, et al., 2005) leading to increased vascular permeability (Lytinas, et al., 2003) and disruption of the BBB (Theoharides and Konstantinidou,

2007) through brain MC activation. (Esposito, et al., 2002) and permitting potential toxins and other environmental triggers to enter the brain.

Our present findings support previous reports of elevated CSF concentrations of SP in FMS patients. (Russell, et al., 1994) Substance P is an eleven amino acid peptide originally isolated and characterized from the rat brain (Leeman and Ferguson, 2000) and has been involved in the pathogenesis of inflammation. (Saraceno, et al., 2006; Douglas and Leeman, 2011; O'Connor, et al., 2004; Steinhoff, et al., 2014; Richard, et al., 2015; Munoz and Covenas, 2014) The neurokinin-1 (NK-1) receptor for SP has been implicated in the pathophysiology of pain, but blockade of that receptor does not control the pain of FMS. (Greenwood-Van, et al., 2014; Russell, 2002) Substance P can stimulate a number of immune cells, especially MCs (Fewtrell, et al., 1982), to release TNF. (Ansel, et al., 1993; Okayama, et al., 1998 ; Okabe, et al., 2000; Theoharides, et al., 2010c; Theoharides, et al., 2010a) Moreover, SP can induce MCs to express CRHR-1 leading to an augmentation of the physiologic stress response. (Asadi, et al., 2012) It should be pointed out that depending when CRH is released and where in the brain, it may not necessarily activate the HPA axis and lead to increased serum cortisol. This may be the reason why serum morning cortisol is not different between FMS patients and controls, but evening cortisol is actually increased in FMS patients. (Fatima, et al., 2013)

It was recently reported that MCs can secrete HK-1, which augments their allergic stimulation.(Sumpter, et al., 2015) HK-1 is also expressed by other immune cells. (Klassert, et al., 2008) Both SP and HK-1 also help generate Th-17 by inducing IL-1β and TNF in monocytes. (Cunin, et al., 2011) HK-1 was shown to contract the human bronchi through activation of both NK-1 and NK-2 receptors. (Grassin-Delyle, et al., 2010)

Even though the effect of CRH on the skin was augmented by NT, (Donelan, et al., 2006a) NT was not increased in the serum of FMS patients. However, no one to date has studied the effect of CRH on MCs stimulation by SP.

Our data has shown that the mean serum levels of the inflammatory cytokine IL-6 are significantly elevated in FMS. There is increasing evidence that changes in this inflammatory cytokine levels may influence the severity of symptoms in FMS patients. (Carvalho, et al., 2008; Nugraha, et al., 2013) In agreement with our findings, other studies reported that IL-6 was increased in the serum of FMS patients (Hernandez, et al., 2010; Behm, et al., 2012; Ross, et al., 2010) and that its levels correlated with FMS severity. (Uceyler, et al., 2011) (Table 2) Nevertheless, some papers reported no difference in serum IL-6 levels between FMS patients and controls. (Gur, et al., 2002; Kim, et al., 2010; Wallace, et al., 2001) It should be, however, noted that the serum levels in the control subjects in these studies were unusually high (e.g. 5.46±1.38 pg/mL, (Gur, et al., 2002) compared to our controls (0.92±0.31 pg/mL) or those reported by Hernadez et al. (0.92±0.32 pg/mL). (Hernandez, et al., 2010)

The increased IL-6 levels may derive from MCs because acute restraint physiological stress of mice led to increased serum IL-6, which was entirely MC-dependent. (Geiss, et al., 2011) Moreover, increased serum IL-6 was associated with bone pain in patients with mastocytosis,(Theoharides, et al., 2015f) who often present with comorbid FMS. (Theoharides, et al., 2015f; Jennings, et al., 2014) It is important to note that patients with mastocytosis experience diffuse musculoskeletal pain (Delsignore, et al., 1996) and are often diagnosed with FMS. (Theoharides, et al., 2015c)

In the present study, we also found significantly increased serum TNF levels in patients with FMS in comparison to healthy controls. One previous study (using ELISA) also reported

increased plasma TNF in FMS patients, (Bazzichi, et al., 2007a) but other studies apparently found no difference in TNF serum levels between FMS patients and controls. (Wallace, et al., 2001; Garcia-Campayo, et al., 2008; Ross, et al., 2010) Surprisingly, another study reported decreased serum TNF levels in FMS patients as compared to controls, but the levels in both patients (20.42±7.24 pg/mL) and controls (35.73±0.72 pg/mL) were 20 times or more higher (Hernandez, et al., 2010) than the levels we report (1.79±0.62 pg/mL and 0.69±0.16 pg/mL, respectively).

Several studies have reported elevated levels of the pro-inflammatory chemokine IL-8 (CXCL8) in both serum (Wallace, et al., 2001; Gur, et al., 2002; Ross, et al., 2010; Rodriguez-Pinto, et al., 2014; Kadetoff, et al., 2012; Bote, et al., 2012) and CSF (Kadetoff, et al., 2012) of patients with FMS (Table 2). Interestingly, one study reported that moderate (45 minutes cycling) exercise *decreased* the serum concentration of IL-8 in FMS patients, but *increased* it in healthy controls; there was also a decrease in the release of IL-6 and TNF from stimulated peripheral monocytes, in FMS patients as compared to healthy women. (Bote, et al., 2013) These finding may help to explain the apparent beneficial effect of mild exercise in FMS

One study showed a strong correlation between increased plasma levels of IL-17A and TNF in patients with FMS. (Pernambuco, et al., 2013) CSF and serum IL-17 also positively correlated with measures of subjective pain among FMS patients, (Meng, et al., 2013) depression and anxiety in patients with rheumatoid arthritis (Liu, et al., 2012) symptoms reported by 30-40% of patients with FMS. TNF and IL-17 seem to act together in perpetuating the inflammatory process. (Romero-Sanchez, et al., 2011; Griffin, et al., 2012) MC-derived IL-6 and TGFβ induce the development of Th-17 cells through dendritic cell maturation. (Dudeck, et al., 2011) Moreover, MCs can secrete IL-17, themselves, (Kenna and Brown, 2013) as shown both in

arthritic joints (Hueber, et al., 2010) and in psoriatic lesions.(Lin, et al., 2011)

We also measured IL-31 because it has been involved in allergies (Rabenhorst and Hartmann, 2014) and IL-33 because it is involved in autoimmunity and inflammation. (Theoharides, et al., 2015a) The serum levels of IL-31 and IL-33 were significantly lower in the FMS group compared to healthy controls.

It is obvious from the previous publications discussed, some of which have been reviewed before (Rodriguez-Pinto, et al., 2014) and are summarized in Table 2, that blood levels of cytokines vary considerably. There are a number of variables that could influence the levels of these blood biomarkers: (a) different assay methods, (b) pre- or post- menopausal female subjects, (c) differences in Bodily Mass Index (BMI), (d) serum or plasma levels may not correspond to or accurately reflect tissue or circulating blood cell cytokine expression or release, (e) may be affected by concurrent medications, and (f) serum cytokine levels may vary if obtained at different times of the day. For instance, it was recently reported that MCs release their mediators in a circadian mode. (Nakamura, et al., 2014) Multiple cytokine testing, rather than single measurement, especially at different times of the day, could be achieved by measuring them in 24 hr urine as it is done with MC mediator metabolites. (Divekar and Butterfield, 2015) In addition, measurements early and late in the course of FMS might prove to be useful. Finally, measuring gene expression of cytokines in relevant tissues maybe more accurate than blood levels.

We recently reviewed the pathophysiology of FMS (Theoharides, et al., 2015e) and the relative lack of reliable biomarkers or effective treatments. We now show that the neuropeptides CRH, SP and HK-1 are significantly elevated in the serum of FMS patients. These molecules could be released centrally and may cause focal inflammation leading to MC activation and pain.

(Heron and Dubayle, 2013) Peripheral inflammation may still have central effects. (Lampa, et al., 2012)

MCs and their mediators have been implicated in neuropsychiatric diseases (Theoharides, et al., 2004), IC/PBS (Sant, et al., 2007) and IBS. (Theoharides, et al., 2012) Increased number of activated MCs was also reported in skin biopsies of FMS patients. (Blanco, et al., 2010) MCs are located perivascularly close to neurons especially in the leptomeninges (Polyzoidis, et al., 2015), thalamus and hypothalamus. (Pang, et al., 1996) We hypothesize that environmental, immune and infectious triggers stimulate MCs leading to secretion of inflammatory mediators (Fig. 1). Stimulated MCs secrete vasoactive and pro-inflammatory mediators such as the preformed heparin, histamine, serotonin, proteases (e.g. tryptase) and TNF, as well as the *de novo* synthesized leukotrienes, prostaglandins, and cytokines (IL-1, IL-6, IL-8, TNF). At least histamine, prostaglandin D_2 (PDG_2) and tryptase are known to stimulate sensory nerves and elicit pain. (Dai, et al., 2004; Chatterjea and Martinov, 2014) In addition, histamine, PGD_2, IL-6, TNF and tryptase could also stimulate microglia, as well as CRH release from the hypothalamus. (Turnbull and Rivier, 1999) MC-microglia interactions are implicated in the pathogenesis of neuroinflammation. (Skaper, et al., 2012) In fact, CRH and microglia have been involved in the pathogenesis of mental disorders. (Kritas, et al., 2014)

Brain cells activated by these mediators or by CRH could then release HK-1 and SP, further stimulating MCs. Brain MCs were reported to synthesize and release TNF. (Cocchiara, et al., 1999) It is particularly important that MCs are the only immune cells that store *pre-formed* TNF, from which it can be secreted rapidly (Zhang, et al., 2011) and superactivate T cells. (Nakae, et al., 2006; Kempuraj, et al., 2008)

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MCs derive from bone marrow progenitors and mature in tissues depending on microenvironmental conditions. (Galli, 1990) MCs are critical for the development of allergic reactions, (Theoharides, et al., 2015f) but are also implicated in immunity (Galli, et al., 2008a) and inflammation. (Theoharides, et al., 2010a; Theoharides, et al., 2015d) We have shown that certain natural flavonoids, (Middleton, et al., 2000) such as luteolin and quercetin (combination of which are found that in the dietary supplement FibroProtek®) can inhibit IL-6 (Kandere-Grzybowska, et al., 2006) and TNF (Kempuraj, et al., 2005; Weng, et al., 2014) release from stimulated MCs. Luteolin and quercetin could, therefore, be useful in FMS (Fig. 5). It is interesting that vitamin D inhibits MCs (Baroni, et al., 2007) and has been reported to regulate in inflammatory diseases. (Toniato, et al., 2015; Calton, et al., 2015)

Conclusion

Our findings of significantly elevated serum levels of the neuropeptides CRH, SP, and its structurally related Hemokinin-1 (HK-1), as well as the cytokines IL-6 and TNF, in FMS patients compared to healthy controls may prove to be diagnostically useful. Preventing the secretion of CRH, SP and/or HK-1 and their ability to stimulate release of IL-6 and TNF from MCs, microglia or other immune cells or may constitute potential new treatment approaches for FMS.

Competing interests

The authors declare that they have no competing interests.

Collection of the FMS samples was accomplished by IJR in the course of an Investigator Initiated Research (IIR) study, financially suported by Pfizer through an institutional grant.

Disclosure

TCT is the inventor of US patents No. 7,906,153; No. 8,268,365 and PCT application No. 13/722, 397 for the treatment of neuroinflammatory conditions.

Authorship contributions

The diagnosis of the FMS, the clinical evaluation of the FMS patients, and the collection of the serum samples from the FMS patients: IJR

Participated in research design: IT, TCT, IJR

Conducted experiments: IT

Performed data analysis: IT

Writing the manuscript: TCT, IT, JMS, RMG and IJR

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Footnotes

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Figure Legends

Figure 1. (A, B) Comparison of serum CRH and SP levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

Figure 2. (A) Comparison of serum HK-1 levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group. **(B) Correlation between serum HK-1 and SP levels.**

Figure 3. (A, B) Comparison of serum IL-6 and TNF levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

Figure 4. (A, B) Comparison of serum IL-33 and IL-31 levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

Figure 5. Diagrammatic representation of the proposed interactions among neuropeptides, mast cells, inflammatory cytokines, neurons and FMS pathogenesis. Environmental, immune and infectious triggers activate MCs leading to secretion of inflammatory mediators such as IL-6 and TNF which could further stimulate nerves to release CRH, HK-1 and SP, further stimulating MCs. Luteolin and quercetin could be of help by blocking MC stimulation and/or release of inflammatory mediators.

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Table 1. Demographic Characteristics of the Human Subject Participants

Characteristics	Fibromyalgia Syndrome (n=84)	Healthy Controls (n=20)
Gender (% female)	100 %	75 %
Age Range (mean years)	28-64 (47.94±11.18)	25-65 (38.15±10.62)
Ethnicity	90 % Caucasian	100 % Caucasian
Pain Severity (mean PVAS)	67.7 mm	non-applicable

PVAS = Pain Visual Analog Scale

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Table 2	Reported cytokines/chemokines levels in FMS			
Cytokines	**Assays**	**N***	**Results**	**References**
IL-6, TNF-α (Serum)	ELISA	54:21 (IL-6) 43:17 (TNF-α)	IL-6, TNF-α ↑	Present results
IL-4, IL-6, IL-8, TNF-α, (Serum)	Bio-Plex cytokine assay	20:80	IL-8, TNF-α ↑ IL-6 ↓ IL-4, IL-10 →	(Wang, et al., 2008)
IL-6, IL-8, TNF-α (Serum)	Luminex	56:32	IL-8 ↓ IL-6, TNF-α → IL-6, IL-8, TNF-α ↓ in older FM patients	(Garcia-Lozano, et al., 2008)
IL-1, IL-6, IL-8 (Serum)	ELISA	81:32	IL-8 ↑ IL-1, IL-6 →	(Gur, et al., 2002)
IL-8 (Serum)	ELISA	20:20	IL-8 ↑	(Bote, et al., 2012)
IL-1α, IL-1β, IL-6, IL-8, TNF-α (Serum)	Luminex	7:12 (non-responders #)	IL-6, IL-8↑	(Ross, et al., 2010)
IL-1β, IL-6, IL-8 (Serum)	ELISA	27:29	IL-8 ↑ IL-1β, IL-6 →	(Kim, et al., 2010)
IL-1β, IL-6, TNF-α (Serum)	ELISA	64:25	IL-6 ↑ IL-1β, TNF-α ↓	(Hernandez, et al., 2010)
IL-6, IL-8 (Serum)	ELISA	105:61	IL-8, IL-6 →	(Xiao, et al., 2013)
IL-1β IL-6, IL-8, TNF-α (Serum)	ELISA	56:36	IL-8 ↑ IL-1β, IL-6, TNF-α →	(Wallace, et al., 2001)
IL-1b, IL-5, IL-6, IL-8, TNF-α (Serum)	ELISA	15:15	IL-8 ↑ IL-1β →	(Kadetoff, et al., 2012)
IL-1, IL-6, IL-8, TNF- α (Plasma)	ELISA	80:45	IL-8, TNF-α ↑	(Bazzichi, et al., 2007b)
IL-6, IL-8, (Plasma)	Luminex	39:47 (IL-6) 62:43 (IL-8)	IL-6, IL-8 ↓	(Behm, et al., 2012)

*no of FMS patients: no of healthy controls; # non-responders = did not increase growth hormone levels after exercise

Fig. 1

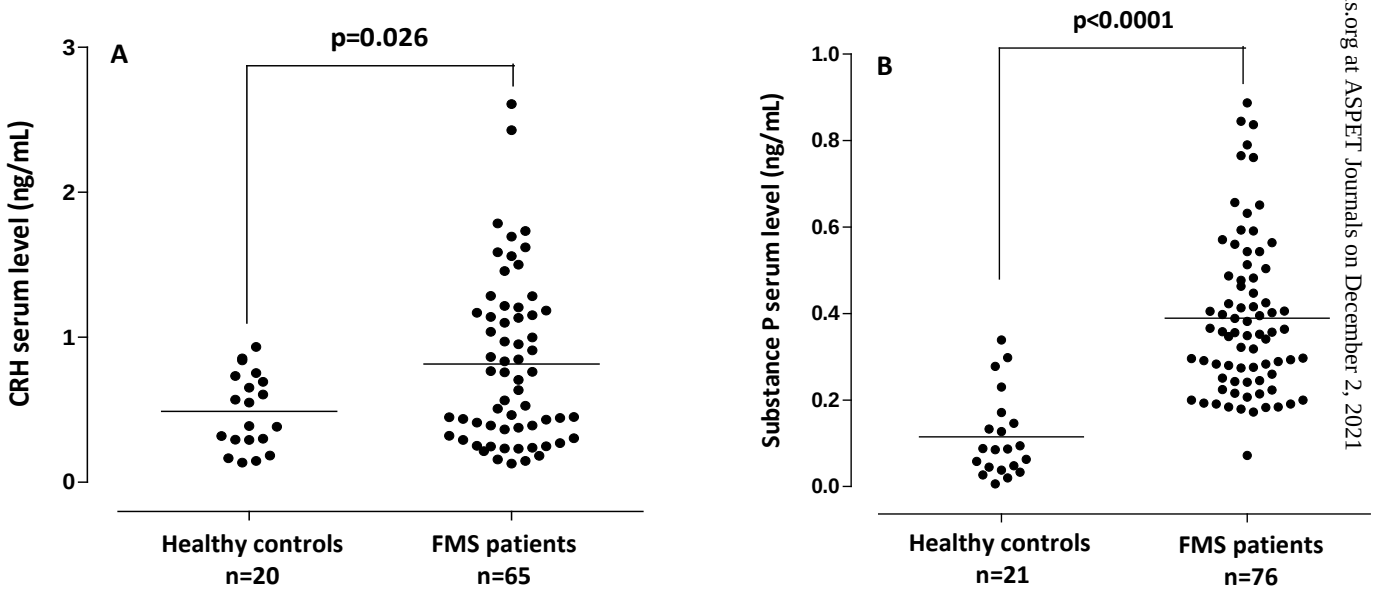

Fig.2

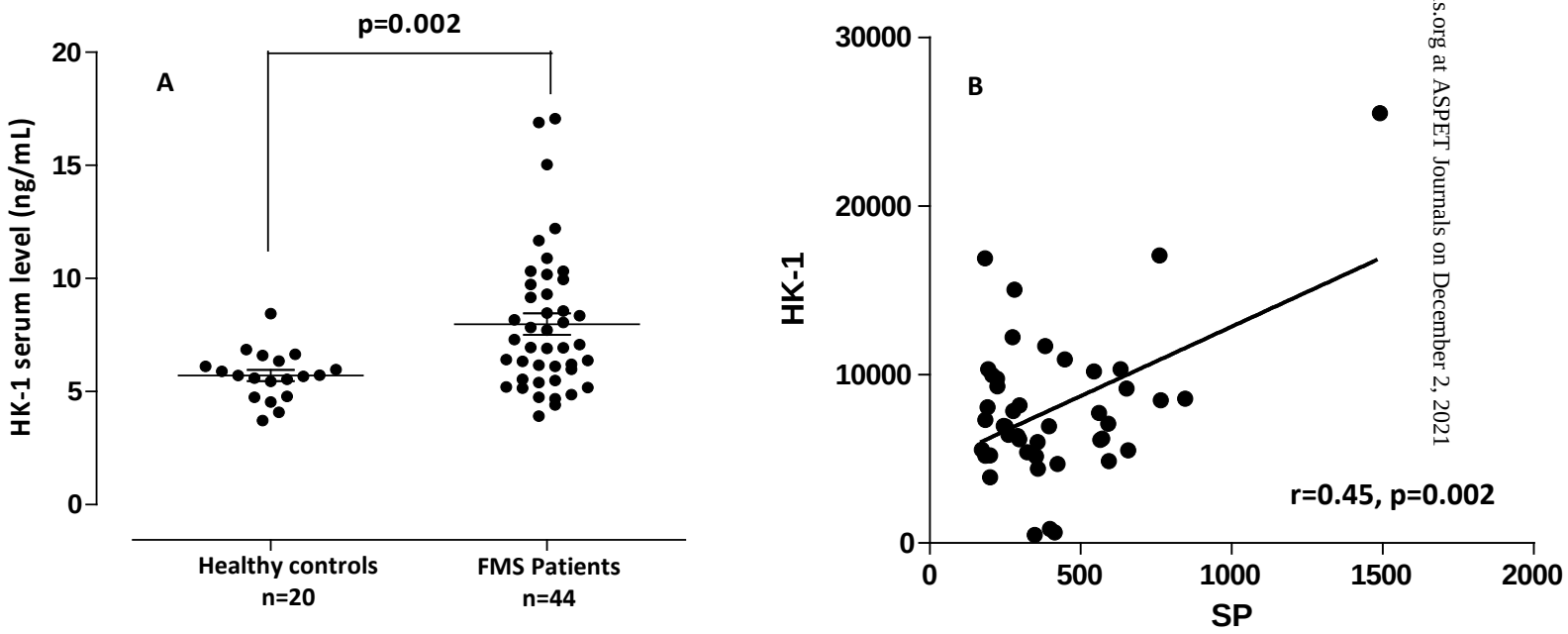

Fig. 3

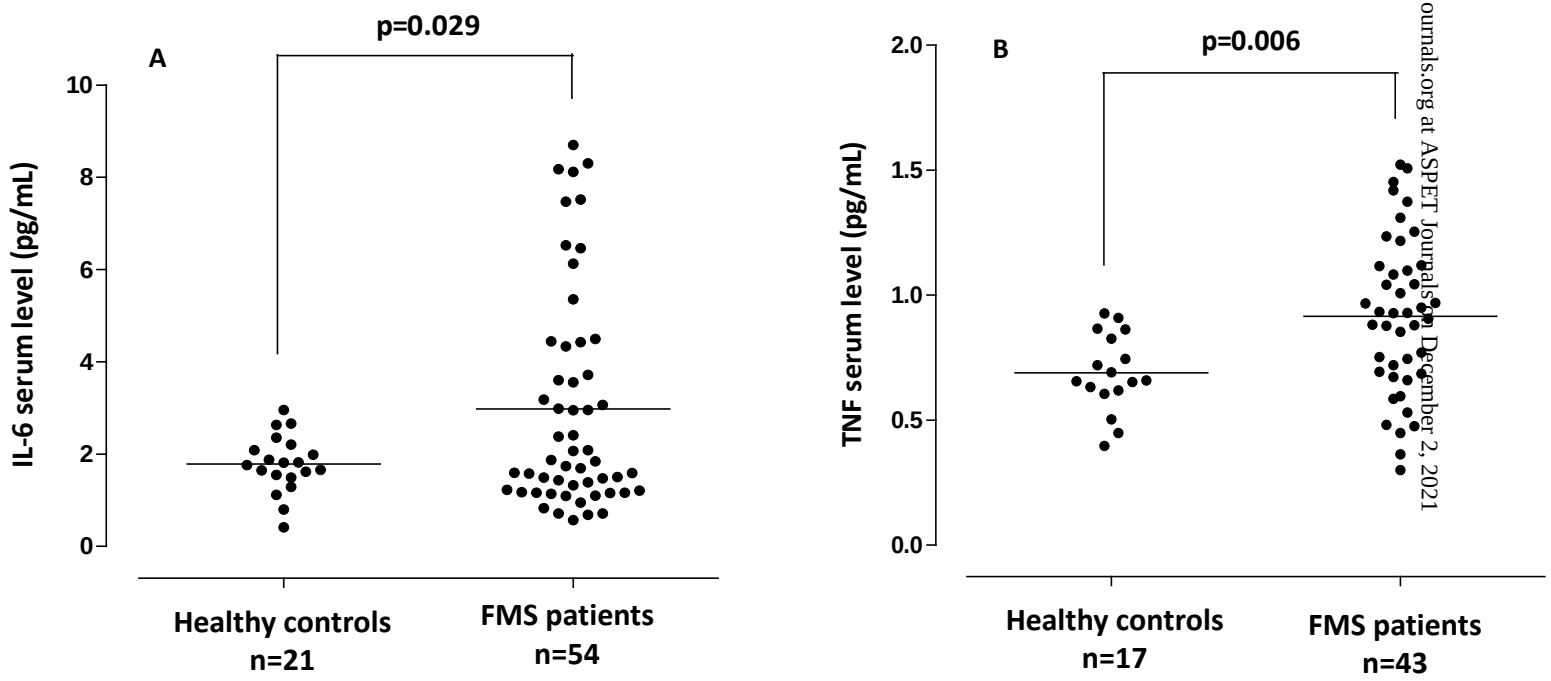

Fig. 4