CHAPTER 3

Basal inflammation and innate immune response in chronic multi-site musculoskeletal pain

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Abstract

Introduction Dysregulation of the immune system may play a role in chronic pain although study findings are inconsistent. This cross-sectional study examined whether basal inflammatory markers and the innate immune response are associated with the presence and severity of chronic multi-site musculoskeletal pain.

Methods Data were used on 1632 subjects of the Netherlands Study of Depression and Anxiety. The Chronic Pain Grade questionnaire was used to determine the presence and severity of chronic multi-site musculoskeletal pain. Subjects were categorized in a chronic multi-site musculoskeletal pain group (n=754) and a control group (n=878). Blood levels of the basal inflammatory markers C-reactive protein, interleukin-6 and tumor necrosis factor-alpha were determined. To obtain a measure of the innate immune response, 13 inflammatory markers were assessed after lipopolysaccharide (LPS) stimulation in a subsample (n=707).

Results Subjects with chronic multi-site musculoskeletal pain showed elevated levels of basal inflammatory markers compared with controls, but statistical significance was lost after adjustment for lifestyle and disease variables. For some LPS-stimulated inflammatory markers, we did find elevated levels in subjects with chronic multi-site musculoskeletal pain both before and after adjustment for covariates. Pain severity was not associated with inflammation within chronic pain subjects.

Conclusions An enhanced innate immune response in chronic multi-site musculoskeletal pain may be examined as a potential biomarker for the onset or perpetuation of chronic pain.

Introduction

Chronic pain, defined as pain lasting longer than three months[1], is prevalent with worldwide population estimates of approximately 10%[2]. Chronic pain usually presents at multiple body locations, typically in the musculoskeletal system[3-5]. Compared to single-site pain, multi-site musculoskeletal pain has been associated with a greater negative impact on patients' functioning[6] and disability[5,7], and an increased risk of depressive and anxiety disorders[8]. Interventions of chronic pain are at best moderately effective[9,10] and, although studying the etiology of chronic pain has gained more attention, its underlying biological mechanisms are only partially understood.

Whereas acute pain can often be attributed to damage of peripheral structures, chronic multi-site musculoskeletal pain is likely to be a result of amplification of nociceptive transmission that can occur without any nociceptive input[1,11], i.e. 'central sensitization'[12]. In central sensitization, dysregulation of cytokine levels may play a role in initiating or perpetuating pain[13,14]. Animal studies suggest that pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) induce central sensitization and contribute to pain hypersensitivity[15,16]. Therefore, inhibition of pro-inflammatory cytokines might be beneficial for the treatment of chronic pain[17].

Although studies on inflammation in chronic multi-site musculoskeletal pain are rare, some studies have investigated cytokine levels in fibromyalgia patients. A recent systematic review indicated elevated IL-1, IL-6 and IL-8 levels in blood serum and normal levels of other cytokines such as TNF-α in fibromyalgia patients[18]. Some studies found unaltered[18] or reduced levels[19] of anti-inflammatory markers IL-4 and IL-10 in blood serum of patients with chronic widespread pain. Although studies on C-reactive protein (CRP) in chronic multi-site musculoskeletal pain are currently lacking, elevated levels of CRP in fibromyalgia have been demonstrated[20-22]. In addition, increased inflammatory levels have previously been associated with higher pain severity in fibromyalgia[22].

Some researchers suggest that immune disturbances may only be revealed after stimulation of the immune system[23]. Alterations in basal inflammatory levels may be harder to detect because they generally have low values and show circadian rhythmicity and large variability[18]. Some studies propose that in fibromyalgia, the innate capacity of the immune system to produce cytokines is disturbed[24,25]. One study among 110 fibromyalgia patients found reduced responses of 8 different cytokines to a mitogen challenge of peripheral blood mononuclear cells (PBMC), compared to 90 healthy controls[24]. Other studies demonstrated enhanced immune responsiveness to stimulation in fibromyalgia patients[23,26,27]. For example, after lipopolysaccharide (LPS) stimulation increased pro-inflammatory IL-1β release by peripheral blood mononuclear cells was found in 19 chronic pain patients, compared with 11 pain-free controls[23].

The few previous studies mostly focused on basal inflammatory levels in fibromyalgia, only assessed a small range of cytokines, had small sample sizes (n<140 [18], with the exception of 2 studies[28,29]; n=425 in largest study[28]), or insufficiently controlled for covariates that may influence immune activity[18,23,24,30]. Therefore, this cross-sectional study investigated the association between basal and LPS-stimulated inflammatory markers, and the presence and severity of chronic multi-site musculoskeletal pain while controlling for sociodemographics, lifestyle and disease variables, depression and anxiety, and medication intake. We hypothesize that elevated levels of inflammatory markers are associated with the presence and severity of chronic multi-site musculoskeletal pain.

Methods

Subjects

The current cross-sectional study was based on data from the Netherlands Study of Depression and Anxiety (NESDA), an ongoing cohort study conducted among 2981 adults, who were between 18 and 65 years of age at the baseline assessment. Subjects were recruited from the general population (n=564), general practices (n=1610) and mental health care organizations (n=807). People in different developmental stages of psychopathology, as well as controls with no psychiatric diagnosis, participated in the study. The NESDA study contains a high proportion of subjects with chronic multi-site musculoskeletal pain and provides a unique opportunity for rigorous control of relevant variables such as depressive and anxiety disorders. Further details of the NESDA study have been described elsewhere[31]. The research protocol was approved by the Ethical Committee of participating universities, and all respondents provided written informed consent.

From the initial 2981 respondents, 767 persons met the criteria for chronic multi-site musculoskeletal pain, and 887 persons for the control group (criteria discussed in measurement section below). Of these 1654 subjects, 22 persons were excluded because no valid data of basal inflammatory markers were available, leaving a total of 1632 subjects for our analyses on basal inflammation (754 with chronic multi-site musculoskeletal pain and 878 controls).

Data of LPS-stimulated inflammatory markers were available for 707 subjects (307 with chronic multi-site musculoskeletal pain and 400 controls). Excluded persons (n=925) from the LPS substudy did not differ in sociodemographic factors, basal inflammatory marker levels, and chronic multi-site musculoskeletal pain, but more often had a lifetime depressive and/or anxiety disorder (76.6 vs. 70.0%, p=0.004) compared with included persons.

Chronic multi-site musculoskeletal pain

Chronic multi-site musculoskeletal pain was measured using the Chronic Pain Grade (CPG)[32], a reliable and valid measure of severity of chronic pain[33,34]. The CPG first inquires about the presence of pain in several locations (ie, the extremities [joints of the arms, hands, legs or feet], back, neck, head, abdomen, chest, and the mouth and face [orofacial area]) in the prior 6 months. The subsequent questions in the CPG refer to the most painful location and include: (1) days in pain in the prior 6 months (scale 0-180); (2) pain at this moment (scale 0-10); (3) worst pain in the prior 6 months (scale 0-10); (4) average pain in the prior 6 months (scale 0-10); (5) disability days in the prior 6 months (scale 0-180); (6) disability in daily activities (scale 0-10); (7) disability in spare time, social life, and family activities (scale 0-10); and (8) disability in work (scale 0-10). According to the CPG protocol, five grades were categorized from these measures: grade 0 (pain-free, no pain in the prior 6 months); grade I (low disability, low intensity); grade II (low disability, high intensity); grade III (high disability, moderately limiting); and grade IV (high disability, severely limiting).

Subjects were classified as having chronic multi-site musculoskeletal pain if they had grade I, II, III or IV and pain present in the extremities, and the back and the neck (n=754). We also refer to the chronic multi-site musculoskeletal pain group as the chronic pain group. The control group consisted of people with grade 0 (n=168) or with grade I and pain in, at most, 2 locations (n=710). The remaining subjects who did not meet the criteria of the chronic multi-site musculoskeletal pain group or the control group were not included in the present study (n=1349).

To indicate pain severity, both pain intensity and pain disability were assessed in subjects with chronic multi-site musculoskeletal pain. For assessment of pain intensity, questions 2, 3 and 4 of the CPG were used (see above) to yield a total pain intensity score (average of the 0-10 ratings of the 3 questions multiplied by 10 resulting in a 0-100 score)[32]. For assessment of pain disability in subjects with chronic multi-site musculoskeletal pain, questions 6, 7 and 8 of the CPG were used (see above) to yield a total pain disability score (average of the 0-10 ratings of the 3 questions multiplied by 10 resulting in a 0-100 score)[32].

Immune system

Basal inflammatory markers included CRP and the pro-inflammatory cytokines IL-6 and TNF-α. Fasting blood samples were obtained in the morning between 8 and 9 am after overnight fasting and kept frozen at –80°C. CRP and IL-6 were assayed at the Clinical Chemistry department of the VU University Medical Center (Amsterdam, The Netherlands). Plasma levels of CRP were measured in duplicate by a using high-sensitivity in-house enzyme-linked immunosorbent assay (ELISA) based on purified protein and polyclonal anti-CRP antibodies (Dako, Glostrup, Denmark). Plasma IL-6 levels were

measured in duplicate by a high sensitivity ELISA (PeliKine CompactTM ELISA, Sanquin, Amsterdam, The Netherlands). Plasma TNF-α levels were assayed in duplicate at Good Biomarker Science (Leiden, The Netherlands) using a high-sensitivity solid phase ELISA (Quantikine HS Human TNF-α Immunoassay, R&D systems, Minneapolis, MN, USA). Intra- and interassay coefficients of variation were 5% and 10% for CRP, 8% and 12% for IL-6, and 10% and 15% for TNF-α, respectively. Furthermore, a basal summary index was calculated as the standardized sum of all 3 standardized ln-transformed basal inflammatory markers.

To reflect the innate production capacity of inflammatory markers, the innate immune response of 17 cytokines was assessed in blood that was ex vivo stimulated with LPS. LPS-stimulated blood was available for 1241 persons of the total NESDA sample. Serial venous whole blood samples were obtained in one 7-mL heparin-coated tube (Greiner Bio-one, Monroe, NC, USA). Between 10 and 60 min after blood draw, 2.5 mL of blood was transferred into a PAXgen tube (Qiagen, Valencia, CA, USA). Remaining blood (4.5 mL) was stimulated by addition of LPS (10 ng/ml blood; *Escherichia coli*, Sigma, St. Louis, MO, USA). LPS-stimulated samples were laid flat and incubated at a slow rotation for 5–6 hours at 37°C. A 2.5-mL sample of this LPS-stimulated blood was transferred into a PAXgene tube (Qiagen). Remaining plasma (±0.5 mL) was kept frozen at –80°C for later assaying. Using a multianalyte profile (Human CytokineMAP A V.1.0; Myriad RBM, Austin, TX, USA) levels of granulocyte-macrophage colony-stimulating factor, interferon-gamma (IFN-γ), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-18, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1α), macrophage inflammatory protein-1beta (MIP-1β), matrix metalloproteinase-2 (MMP-2), TNF-α and TNF-β were assayed. For granulocyte-macrophage colony-stimulating factor, IL-3, IL-5 and IL-7, too little valid values were obtained (<200) and were therefore excluded from further analyses, leaving a total of 13 cytokines. In addition, an LPS summary index was calculated as the standardized sum of all 13 standardized markers.

Covariates

Basic covariates included sociodemographic characteristics (age, sex, and years of education). In addition, several 'lifestyle and disease variables' were assessed as these have previously been associated with both inflammation and chronic pain. Body mass index was calculated as body weight in kilograms divided by height in meters squared. Smoking was categorized as never smoker, former smoker and current smoker. Alcohol use was categorized as non-drinker, mild/moderate drinker (1–21 glasses/week for men and 1–14 glasses/week for women), and heavy drinker (>21 glasses/week for men and >14 glasses/week for women)[35]. Physical activity was assessed with the International Physical Activity Questionnaire[36] and expressed in 1000 metabolic equivalent

minutes per week. Chronic diseases (including cardiovascular disease, epilepsy, diabetes mellitus, osteoarthritis, stroke, cancer, chronic lung disease, thyroid disease, liver disease, intestinal disorders, and ulcers) were assessed by self-report and considered present if persons were under treatment. Medication use was assessed based on drug container inspection of all drugs used in the past month and classified according to the World Health Organization (WHO) Anatomical Therapeutic Chemical (ATC) classification[37]. Frequent use (daily or >50% of the time) of anti-inflammatory medication was considered and included ATC codes M01A, M01B, A07EB and A07EC.

In addition, depressive and anxiety disorders and use of antidepressant medication were considered as covariates, as these have previously been related to inflammation[38] and pain[8,39]. Lifetime depressive and anxiety disorders were established with the Composite International Diagnostic Interview (WHO version 2.1) according to Diagnostic and Statistical Manual, 4th edition criteria[40]. Frequent use (daily or >50% of the time) of antidepressant medication included tricyclic antidepressants (ATC code N06AA), selective serotonin reuptake inhibitors (ATC code N06AB) and other antidepressant medications (ATC codes N06AF/AG/AX).

Statistical analyses

Descriptive baseline characteristics were reported as means or percentages in the chronic multi-site musculoskeletal pain and control groups. For the examination of differences between groups, independent-sample *t*-tests were used for continuous variables and χ^2 tests for dichotomous and categorical variables. Pearson's correlations were calculated to examine the associations between basal and LPS-stimulated inflammatory markers. Also, associations between inflammatory markers and covariates were examined using Pearson's correlations and analyses of variance.

For all inflammatory markers, (adjusted) means across controls and chronic pain subjects were calculated. The difference between controls and chronic pain subjects was examined using analyses of (co)variance. To obtain a measure of effect size, Hedges' *g* was calculated for the 2 inflammatory summary indexes. To normalize distributions, all inflammatory marker levels, except LPS-stimulated MMP-2 and LPS-stimulated TNF-β, were ln-transformed prior to analyses. Back-transformed values are presented in the Tables to aid interpretation. Associations between inflammatory markers and the severity of chronic pain were tested using linear regression analyses with inflammatory markers as dependent variables within the subgroup of persons with chronic pain (n=754). Four models were tested: 1) unadjusted (for analyses of LPS-stimulated inflammatory markers: adjusted for laboratory site); 2) additionally adjusted for sociodemographic variables, ie, sex, age, and education; 3) additionally adjusted for lifestyle and disease variables, ie, alcohol use, smoking, body mass index,

number of chronic diseases, physical activity and use of anti-inflammatory medication, and; 4) additionally adjusted for lifetime diagnoses of depressive and anxiety disorders and use of antidepressants. The change in the unstandardized beta-coefficient (B) of the association between inflammation and chronic pain was examined to determine the degree to which covariates influenced the association between inflammation and chronic pain. Since we analyzed correlated measures, analyses were not corrected for multiple statistical testing[41]. For all statistical tests, a probability level of ≤5% was regarded as significant. The statistical calculations were performed using SPSS, version 20.0 (IBM, Armonk, NY, USA).

Results

Baseline characteristics

Compared to controls, subjects with chronic multi-site musculoskeletal pain were significantly older, were more often women, had less education, had a higher body mass index, were more often current smokers, were more frequently non-alcohol drinkers, were more physically active, had more chronic diseases, more frequently used anti-inflammatory medication, more often had a lifetime depressive and/or anxiety disorder, and more frequently used antidepressants (all $p<0.05$, Table 1).

Pearson's intercorrelations for basal inflammatory markers were small (r range from 0.11 to 0.31; see Supplementary Table 1 at the end of this chapter). Pearson's intercorrelations for most LPS-stimulated inflammatory markers were $r>.50$, with somewhat lower correlations for IL-4, IL-8, IL-10 and IL-18, with other LPS-stimulated inflammatory markers (see Supplementary Table 2). Pearson's correlation between the basal summary index and the LPS summary index was small ($r=0.12$, $p<0.001$) suggesting that these are two distinct indicators of immune regulation. All covariates, with the exception of physical activity, were associated with at least one inflammatory summary index (see Supplementary Table 3). In general, associations between covariates and LPS-stimulated markers were less strong than associations between covariates and basal inflammatory markers. Education, body mass index, number of chronic diseases, alcohol use, and anti-inflammatory medication and antidepressant use were more strongly associated with basal inflammatory markers than with LPS-stimulated markers. Smoking and gender were more strongly associated with LPS-stimulated markers than with basal inflammatory markers. Lifetime diagnoses of depression and anxiety were associated with both basal inflammatory markers and LPS-stimulated markers.

Table 1. Baseline characteristics*

	Controls (n=878)	Chronic pain (n=754)	*P*†
Pain			
Days of pain in the prior 6 months	34.4 ± 52.1	108.1 ± 69.7	<0.001
Pain intensity	27.1 ± 11.2	53.0 ± 17.9	<0.001
Pain disability	12.1 ± 13.9	39.9 ± 25.8	<0.001
Sociodemographic factors			
Age, years	42.1 ± 13.6	44.7 ± 12.1	<0.001
Women, %	56.8	73.3	<0.001
Education, years	13.0 ± 3.3	11.3 ± 3.2	<0.001
Lifestyle and health factors			
Body mass index, kg/m^2	25.0 ± 4.3	26.5 ± 5.2	<0.001
Smoking, %			<0.001
Never smoker	31.4	26.0	
Former smoker	35.6	31.2	
Current smoker	32.9	42.8	
Alcohol use, %			<0.001
Non-drinker	23.1	39.9	
Mild/moderate drinker	64.2	48.8	
Heavy drinker	12.6	11.3	
Physical activity, 1000 MET min/week	3.6 ± 2.9	3.9 ± 3.2	0.02
Number of chronic diseases	0.4 ± 0.7	1.0 ± 1.0	<0.001
Anti-inflammatory medication, %	8.4	26.5	<0.001
Depression and anxiety factors			
Lifetime depressive or anxiety disorder, %			<0.001
No disorder	38.2	11.5	
Depressive disorder	18.2	16.7	
Anxiety disorder	14.1	9.5	
Comorbid depressive/anxiety disorder	29.5	62.2	
Antidepressant medication, %			<0.001
No antidepressant	82.0	68.0	
SSRI	12.0	21.4	
TCA	2.1	2.9	
Other antidepressant	3.9	7.7	

* Values are mean ± SD unless otherwise indicated. † Based on independent-sample *t*-test for continuous variables and χ^2 test for dichotomous and categorical variables. MET, metabolic equivalent; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

Basal inflammatory markers and the presence of chronic multi-site musculoskeletal pain

Table 2 reports (adjusted) mean levels of basal inflammatory markers in controls and chronic multi-site musculoskeletal pain subjects. All 3 basal inflammatory markers and the basal summary index were significantly higher in subjects with chronic pain than controls in the unadjusted model. Levels of basal inflammatory markers, with the exception of TNF-α (p=0.30), remained significantly higher in chronic pain subjects with additional adjustment for sociodemographic variables (basal summary index: Hedges' *g*=0.15, p=0.002). These group differences were no longer significant after adjustment for lifestyle and disease variables. Adjustment for body mass index (ΔB=50%) and number of chronic diseases (ΔB=31%) most importantly weakened the association between the basal summary index

and chronic pain (basal summary index: Hedges' *g*=0.04, p=0.51). These findings were hardly affected by additional adjustment for depression and anxiety factors and remained not significant.

Table 2. Unadjusted and adjusted means* for basal inflammatory levels in controls and subjects with chronic multi-site musculoskeletal pain

	Controls (n=878) Adjusted mean (SE)	**Chronic pain (n=754)** Adjusted mean (SE)	***p***
CRP (mg/L)			
Unadjusted	1.09 (1.04)	1.45 (1.04)	<0.001
Sociodemographic [a]	1.17 (1.04)	1.34 (1.04	0.04
Lifestyle & disease [b]	1.25 (1.04)	1.25 (1.04)	0.91
Depression & anxiety [c]	1.25 (1.04)	1.25 (1.04)	0.98
IL-6 (pg/mL)			
Unadjusted	0.79 (1.02)	0.91 (1.03)	<0.001
Sociodemographic [a]	0.80 (1.02)	0.90 (1.03)	0.002
Lifestyle & disease [b]	0.82 (1.02)	0.87 (1.03)	0.14
Depression & anxiety [c]	0.82 (1.03)	0.87 (1.03)	0.13
TNF-α (pg/mL)			
Unadjusted	0.80 (1.02)	0.86 (1.02)	0.02
Sociodemographic [a]	0.82 (1.02)	0.84 (1.02)	0.30
Lifestyle & disease [b]	0.83 (1.02)	0.83 (1.03)	0.98
Depression & anxiety [c]	0.83 (1.02)	0.84 (1.03)	0.78
Basal summary index			
Unadjusted	-0.13 (0.03)	0.15 (0.04)	<0.001
Sociodemographic [a]	-0.07 (0.03)	0.08 (0.04)	0.002
Lifestyle & disease [b]	-0.02 (0.03)	0.02 (0.04)	0.51
Depression & anxiety [c]	-0.02 (0.03)	0.03 (0.04)	0.38

* Based on analyses of covariance; [a] adjusted for sex, age, years of education; [b] additionally adjusted for alcohol use, smoking, body mass index, number of chronic diseases, physical activity and use of anti-inflammatory medication; [c] additionally adjusted for lifetime diagnoses of depressive and anxiety disorders and use of antidepressants. CRP, C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; basal summary index = standardized sum of all standardized ln-transformed basal inflammatory markers.

LPS-stimulated inflammatory markers and the presence of chronic multi-site musculoskeletal pain

Table 3 shows (adjusted) mean levels of LPS-stimulated inflammatory markers in controls and chronic multi-site musculoskeletal pain subjects in a random subsample (n=707). Unadjusted analyses indicated that chronic pain subjects had higher levels of LPS-stimulated IL-2, IL-8, TNF-β and MMP-2. After adjustment for sociodemographic factors, IL-2, IL-6, IL-8, TNF-α, TNF-β and MMP-2 were significantly higher in chronic pain subjects. After additional adjustment for lifestyle and disease variables, group differences remained significant for IL-2, TNF-β and MMP-2 ($p<0.05$), whereas these differences became not significant for IL-6, IL-8 and TNF-α ($p>0.05$). After adjustment for depression and anxiety factors, IFN-γ, IL-2, TNF-α and TNF-β were significant, whereas MMP-2 became non-significant ($p=0.08$). The LPS-stimulated pro-inflammatory markers IFN-γ, IL-2, and TNF-α were higher in subjects with chronic pain than in control subjects, while the anti-inflammatory markers IL-4 and IL-10 did not significantly differ between the groups with and without chronic pain.

The LPS summary index was significantly elevated in chronic pain subjects compared to controls both before and after adjustment for lifestyle and disease variables (Hedges' g=0.18, p=0.003 after adjustment for sociodemographics). Adjustment for number of chronic diseases (ΔB=17%) and body mass index (ΔB=13%) most importantly weakened the association between the LPS summary index and chronic pain. After adjustment for depression and anxiety factors, the association between the LPS summary index and chronic pain became non-significant (Hedges' g=0.12, p=0.07).

Since depression and anxiety have been associated with elevated inflammatory levels[38,42], we examined whether lifetime diagnoses of depression and anxiety moderated the association between chronic pain and inflammatory markers. We therefore repeated analyses of covariance and added an interaction term (presence of chronic pain*presence of depression/anxiety) as a predictor variable. However, no significant chronic pain-by-depression/anxiety interaction term was found with any of the inflammatory markers (data not shown).

Previous studies suggest that age moderates the association between stress and immune functioning[43]. Therefore, we repeated analyses of variance and added an interaction term (presence of chronic pain*age). No significant chronic pain-by-age interaction term was found for the basal summary index nor for the LPS summary index (data not shown).

Table 3. Unadjusted and adjusted means* for LPS-stimulated inflammatory markers in controls and subjects with chronic multi-site musculoskeletal pain

	Controls(n=400) Adjusted mean (SE)	**Chronic pain (n=307)** Adjusted mean (SE)	***p***
IFN-γ (pg/mL)			
Unadjusted [a]	9.58 (1.03)	10.18 (1.03)	0.25
Sociodemographic [b]	9.49 (1.03)	10.38 (1.04)	0.07
Lifestyle & disease [c]	9.39 (1.03)	10.38 (1.04)	0.06
Depression & anxiety [d]	9.39 (1.03)	10.49 (1.04)	0.04
IL-2 (pg/mL)			
Unadjusted [a]	7.61 (1.03)	10.59 (1.03)	0.008
Sociodemographic [b]	7.61 (1.03)	8.50 (1.03)	0.01
Lifestyle & disease [c]	7.61 (1.03)	8.50 (1.03)	0.02
Depression & anxiety [d]	7.54 (1.03)	8.50 (1.04)	0.02
IL-4 (pg/mL)			
Unadjusted [a]	8.00 (1.04)	8.67 (1.04)	0.11
Sociodemographic [b]	8.08 (1.04)	8.58 (1.04)	0.22
Lifestyle & disease [c]	8.00 (1.04)	8.67 (1.04)	0.15
Depression & anxiety [d]	8.08 (1.04)	8.58 (1.05)	0.32
IL-6 (ng/mL)			
Unadjusted [a]	21.12 (1.03)	22.42 (1.03)	0.17
Sociodemographic [b]	20.70 (1.03)	22.87 (1.03)	0.03
Lifestyle & disease [c]	21.12 (1.03)	22.42 (1.04)	0.24
Depression & anxiety [d]	21.12 (1.03)	22.42 (1.04)	0.20
IL-8 (ng/mL)			
Unadjusted [a]	9.21 (1.03)	10.28 (1.03)	0.01
Sociodemographic [b]	9.21 (1.03)	10.17 (1.03)	0.03
Lifestyle & disease [c]	9.39 (1.03)	9.87 (1.03)	0.30
Depression & anxiety [d]	9.49 (1.03)	9.78 (1.04)	0.52
IL-10 (pg/mL)			
Unadjusted [a]	192.5 (1.04)	204.4 (1.04)	0.25
Sociodemographic [b]	190.6 (1.04)	206.4 (1.04)	0.21
Lifestyle & disease [c]	192.5 (1.04)	204.4 (1.04)	0.40
Depression & anxiety [d]	194.4 (1.04)	202.4 (1.05)	0.58
IL-18 (pg/mL)			
Unadjusted [a]	244.7 (1.02)	249.6 (1.02)	0.23
Sociodemographic [b]	242.3 (1.02)	252.1 (1.03)	0.14
Lifestyle & disease [c]	244.7 (1.02)	249.6 (1.02)	0.37
Depression & anxiety [d]	244.7 (1.02)	249.6 (1.02)	0.59
TNF-α (ng/mL)			
Unadjusted [a]	2.51 (1.03)	2.66 (1.03)	0.25
Sociodemographic [b]	2.46 (1.03)	2.75 (1.03)	0.02
Lifestyle & disease [c]	2.48 (1.03)	2.69 (1.04)	0.09
Depression & anxiety [d]	2.46 (1.03)	2.75 (1.04)	0.04
TNF-β (pg/mL)			
Unadjusted [a]	295.7 (5.87)	328.9 (6.71)	<0.001
Sociodemographic [b]	293.8 (5.91)	331.5 (6.81)	<0.001
Lifestyle & disease [c]	296.7 (6.12)	327.7 (7.12)	0.002
Depression & anxiety [d]	297.4 (6.37)	326.7 (7.48)	0.007

Table 3. Unadjusted and adjusted means* for LPS-stimulated inflammatory markers in controls and subjects with chronic multi-site musculoskeletal pain *(Continued)*

	Controls(n=400) Adjusted mean (SE)	**Chronic pain (n=307)** Adjusted mean (SE)	***p***
MCP-1 (ng/mL)			
Unadjusted [a]	1.35 (1.03)	1.40 (1.03)	0.28
Sociodemographic [b]	1.35 (1.03)	1.39 (1.03)	0.53
Lifestyle & disease [c]	1.38 (1.03)	1.36 (1.03)	0.71
Depression & anxiety [d]	1.39 (1.03)	1.34 (1.04)	0.44
MIP-1α (ng/mL)			
Unadjusted [a]	14.59 (1.03)	15.64 (1.03)	0.17
Sociodemographic [b]	14.44 (1.03)	15.80 (1.04)	0.09
Lifestyle & disease [c]	14.88 (1.03)	15.33 (1.04)	0.55
Depression & anxiety [d]	14.73 (1.03)	15.33 (1.04)	0.44
MIP-1β (ng/mL)			
Unadjusted [a]	204.4 (1.02)	212.7 (1.03)	0.23
Sociodemographic [b]	204.4 (1.02)	214.9 (1.03)	0.11
Lifestyle & disease [c]	206.4 (1.02)	210.6 (1.03)	0.57
Depression & anxiety [d]	206.4 (1.02)	210.6 (1.03)	0.58
MMP-2 (ng/mL)			
Unadjusted [a]	69.27 (0.84)	73.28 (0.96)	0.002
Sociodemographic [b]	69.20 (0.84)	73.37 (0.97)	0.002
Lifestyle & disease [c]	69.61 (0.87)	72.83 (1.02)	0.03
Depression & anxiety [d]	69.82 (0.91)	72.56 (1.07)	0.08
LPS summary index			
Unadjusted [a]	-0.07 (0.04)	0.09 (0.04)	0.008
Sociodemographic [b]	-0.08 (0.04)	0.10 (0.04)	0.003
Lifestyle & disease [c]	-0.06 (0.04)	0.07 (0.05)	0.04
Depression & anxiety [d]	-0.05 (0.04)	0.07 (0.05)	0.07

* Based on analyses of covariance; [a] adjusted for laboratory site; [b] additionally adjusted for sex, age and years of education; [c] additionally adjusted for alcohol use, smoking, body mass index, number of chronic diseases, physical activity and use of anti-inflammatory medication; [d] additionally adjusted for lifetime diagnoses of depressive and anxiety disorders and use of antidepressants. IFN-γ, interferon gamma; IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; LPS summary index = standardized sum of all standardized (ln-transformed) LPS-stimulated inflammatory markers.

Inflammation and the severity of chronic multi-site musculoskeletal pain

Among subjects with chronic multi-site musculoskeletal pain, linear regression analyses showed no significant associations between pain intensity or pain disability with the basal summary index (n=754) nor with the LPS summary index (n=307) after adjustment for lifestyle and health factors (data not shown). Separate analyses for each inflammatory marker showed no significant association with pain severity after adjustment for covariates, with the exception of LPS-stimulated IL-10 (β=–0.12, p=0.02) and LPS-stimulated IFN-γ (β=0.13, p=0.03) with pain disability after adjustment for all confounders (results not shown).

Discussion

The objective of this study was to investigate the association between dysregulation of the immune system and the presence and severity of chronic multi-site musculoskeletal pain. Chronic pain subjects showed elevated levels of basal inflammatory markers, but this association was no longer significant after adjustment for lifestyle and disease variables. Chronic pain subjects showed elevated levels of some LPS-stimulated inflammatory markers, which remained after adjustment for lifestyle and disease variables.

Our findings show that the association between basal inflammatory markers and chronic pain was more strongly influenced by lifestyle and disease factors than the association between LPS-stimulated inflammatory markers and chronic pain. In particular, body mass index appeared to explain the association between basal inflammatory marker levels and chronic pain. Several studies have shown that chronic pain sufferers tend to be overweight[44,45] and that adipose tissue is an important determinant of a low-grade chronic inflammatory state as reflected by modestly elevated levels of IL-6, TNF-α and CRP[46,47]. Moreover, excess body weight has previously been related to worsening of multi-site pain symptoms[48,49]. Previous studies did not take these lifestyle factors into account[18,24,50] and might have misinterpreted the association between increased basal inflammatory levels and fibromyalgia. Our study suggests that obesity may contribute to a mild inflammatory state in chronic pain. Therefore, a healthy lifestyle that reduces overweight may potentially decrease inflammatory levels in patients with chronic pain[51].

Our finding of an increased LPS summary index in chronic multi-site musculoskeletal pain, which remained after adjustment for sociodemographics, lifestyle and disease variables, may indicate an enhanced innate immune response in chronic pain. The innate immune response reflects the production capacity of inflammatory markers and is known to be under strong genetic control[52]. To our knowledge, only one previous study has assessed both basal inflammation and the innate immune response[23]. Kwok et al. found unaltered basal level and increased LPS-stimulated level of IL-1β in

chronic pain patients[23]. Whereas their study was limited to one cytokine, our study was able to expand this finding to a broader range of cytokines. Our findings suggest that the innate immune response could serve as a potential biomarker for chronic pain.

Overall, our findings suggest an upregulated innate inflammatory response in chronic pain. The association with chronic pain was indicated to be stronger for LPS-stimulated pro-inflammatory than anti-inflammatory cytokines. Among the most significant were MMP-2 and the pro-inflammatory cytokines IFN-γ and TNF-α. IFN-γ has been previously linked to fatigue, arthralgias and myalgias, whereas TNF-α has found to play a role in hyperalgesia, myalgia, fatigue, rapid eye movement (REM) sleep and depression[53]. MMP-2 plays a role in the breakdown of extracellular matrix and seems also involved in the arthritis syndrome[54] and peripheral nerve injury[55]. Thus, the enhanced innate immune response that was observed in our study, may reflect a disposition to respond with pro-inflammatory cytokines that may induce central sensitization and contribute to pain hypersensitivity[15,16].

Glial cells might play an important role in the association between immune dysregulation and chronic pain. Some studies suggest that glial cells, releasing pro-inflammatory cytokines, play an important role in the creation and maintenance of pathological pain states[56]. It could be that with prolonged incoming pain signals, glial cells do not return to an inactivated state but remain 'primed'[57]. Subsequently, these 'primed' glial cells become sensitized to over-respond to pain signals, which might result in an ongoing immune response and chronic pain[58]. Therefore, blocking of glial activation has been proposed as pharmacological treatment for chronic pain[59]. In neurological research, glial cells and the accompanying cortical innate immune response were suggested to cause local neuronal excitability leading to epileptic seizures[60]. Although innate immunity likely plays a role in chronic pain, the complete understanding of underlying processes remains to be elucidated, also because cytokines interact with other biological mechanisms involved in chronic pain, such as the hypothalamic-pituitary-adrenal axis and the autonomic nervous system[61-63].

Within the chronic multi-site musculoskeletal pain group, we found no associations of inflammatory markers with pain severity, which may suggest that if subjects are sensitized to chronic pain, the increased activity of the immune system does not further amplify pain severity. Prospective studies should further investigate the possible differential role for immune dysregulation in the onset and perpetuation of chronic pain.

One of the limitations of the current study is the cross-sectional design which does not allow us to draw conclusions regarding causality. Longitudinal studies should investigate whether the enhanced innate immune response is a contributing factor, correlate, or consequence of chronic

multi-site musculoskeletal pain. Second, most previous studies examined chronic widespread pain using diagnostic criteria by Wolfe et al.[64], whereas we examined pain in the extremities, the back and the neck. Extensive multi-site pain can occur without meeting the classification criteria for chronic widespread pain and therefore setting broader parameters for studying biological mechanisms in chronic pain patients might be needed[3,4]. Third, one of the criteria for chronic pain is pain for ≥3 months, whereas we used the CPG that assesses the number of days in pain in the prior 6 months. However, subjects with chronic pain in this study had on average 115 days of pain (~4 months) in the prior 6 months. It is likely that most of our chronic pain subjects met the criteria of pain ≥3 months, and it is unlikely that the slight deviation with regard to the criterion of pain duration explains our findings. Fourth, our study did not assess pain duration beyond the period of 6 months. As physiological responses depend on the duration of stress such as pain[22], future studies could compare inflammatory profiles between persons in the early stage of chronic pain and persons who have lived with the condition for years. Finally, we included persons with grade I on the CPG and pain in ≤2 locations in the control group in order to increase its sample size. Comparisons with a more strict control group might elucidate even stronger associations. However, chronic pain in 1 or 2 locations is quite common in the population[2] which suggests that our control group resembled the general population and even improved the generalizability of our findings.

Nonetheless, our study is one of the first to assess cytokine levels after LPS-stimulation in subjects with chronic multi-site musculoskeletal pain and provides clear evidence that this novel technique of ex vivo stimulation is useful in detecting immune dysregulation in chronic pain. Other strong aspects of this study include a large sample size and the possibility to control for relevant covariates that were rigorously examined in this study.

In summary, the present study showed increased basal inflammatory markers in chronic multi-site musculoskeletal pain compared with controls, but statistical significance was lost after adjustment for lifestyle and disease variables. Therefore, these variables should be taken into account when examining immune function in chronic pain. Moreover, suggestive evidence for an enhanced innate immune response was found in chronic multi-site musculoskeletal pain. Future longitudinal studies should investigate whether an enhanced immune response is associated with the onset or perpetuation of chronic multi-site musculoskeletal pain.

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Supplementary Tables

Supplementary Table 1. Intercorrelations between basal inflammatory markers

	CRP	IL-6	TNF-α
CRP	1		
IL-6	.31**	1	
TNF-α	.13**	.11**	1

Based on Pearson's r. Levels of basal inflammatory markers were ln-transformed prior to analyses.
** Correlation is significant at the 0.01 level

Supplementary Table 2. Intercorrelations between LPS-stimulated inflammatory markers

	IFN-γ	IL-2	IL-4	IL-6	IL-8	IL-10	IL-18	TNF-α	TNF-β	MCP-1	MIP-1α	MIP-1β	MMP-2
IFN-γ	1												
IL-2	.54**	1											
IL-4	.27**	.34**	1										
IL-6	.78**	.62**	.28**	1									
IL-8	.32**	.42**	.34*	.54**	1								
IL-10	.47**	.28**	.09*	.51**	.21**	1							
IL-18	.43**	.42**	.28**	.53**	.51**	.23**	1						
TNF-α	.79**	.53**	.30**	.86**	.43**	.47**	.48**	1					
TNF-β	.64**	.69**	.38**	.73**	.44**	.47**	.52**	.68**	1				
MCP-1	.52**	.45**	.21**	.64**	.61**	.47**	.50**	.51**	.52**	1			
MIP-1α	.66**	.53**	.32**	.81**	.66**	.46**	.52**	.77**	.66**	.69**	1		
MIP-1β	.68**	.50**	.26**	.81**	.53**	.60**	.49**	.76**	.67**	.70**	.93**	1	
MMP-2	.67**	.68**	.37*	.77**	.57**	.49**	.53**	.69**	.81**	.75**	.77**	.76**	1

Based on Pearson's r. Levels of LPS-stimulated inflammatory markers were ln-transformed prior to analyses. ** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

Supplementary Table 3. Correlations and group comparisons for covariates with basal and LPS-stimulated inflammatory markers

	Basal summary index Mean (SD) or *r*	***p***[1]	**LPS summary index** Mean (SD) or *r*	***p***[1]
Age	0.16	<0.001	0.15	<0.001
Sex		0.12		<0.001
Men	–0.05 (0.97)		0.24 (1.00)	
Women	0.03 (1.01)		–0.14 (0.97)	
Education	–0.20	<0.001	–0.12	0.002
Body mass index	0.38	<0.001	0.13	0.001
Smoking status				
Never smoker	–0.06 (1.01)	ref	–0.13 (1.06)	ref
Former smoker	–0.04 (0.99)	0.30	–0.03 (0.96)	0.51
Current smoker	0.08 (1.00)	0.01	0.15 (0.97)	0.004
Alcohol intake				
None	0.22 (1.01)	ref	–0.04 (1.03)	ref
Moderate	–0.13 (0.97)	<0.001	0.01 (0.97)	0.83
Heavy	0.07 (1.04)	0.29	0.07 (1.09)	0.51
Physical activity	0.01	0.84	–0.02	0.66
Number of chronic diseases	0.16	<0.001	0.13	0.001
Anti-inflammatory medication		0.05		0.07
No	–0.02 (0.98)		–0.03 (1.03)	
Yes	0.11 (1.09)		0.15 (0.84)	
Lifetime diagnoses of depression and anxiety		0.009		0.005
No	–0.11 (0.97)		–0.16 (1.04)	
Yes	0.04 (1.01)		0.07 (0.98)	
Antidepressants		<0.001		0.40
No	–0.05 (0.98)		–0.02 (1.01)	
Yes	0.17 (1.03)		0.06 (0.96)	

[1] based on one-way analyses of variance for dichotomous and categorical variables and Pearson's *r* for continuous variables.