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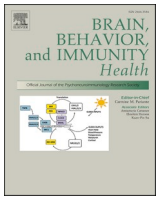

Altered response to Toll-like receptor 4 activation in fibromyalgia: A low-dose, human experimental endotoxemia pilot study

Chloe Jones [a,1], Luke Parkitny [b,c,1], Larissa Strath [d,e], Brant M. Wagener [f], Andrew Barker [f], Jarred Younger [a,*]

[a] Department of Psychology, University of Alabama at Birmingham, 1300 University Blvd, Birmingham, AL, 35294, USA
[b] Departments of Neurology and Pediatrics, Baylor College of Medicine, Houston, TX, USA
[c] Jan and Dan Neurological Research Institute, Texas Children's Hospital, Houston, TX, USA
[d] Pain Research and Intervention Center of Excellence, The University of Florida, Gainesville, FL, USA
[e] College of Medicine, Department of Health Outcomes and Biomedical Informatics, The University of Florida, Gainesville, FL, USA
[f] Department of Anesthesiology and Perioperative Medicine, Division of Critical Care Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

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ABSTRACT

In this pilot study, a human intravenous injection of low-dose endotoxin (lipopolysaccharide, LPS) model was used to test if fibromyalgia is associated with altered immune responses to Toll-like receptor 4 (TLR4) activation. Eight women with moderately-severe fibromyalgia and eight healthy women were administered LPS at 0.1 ng/kg in session one and 0.4 ng/kg in session two. Blood draws were collected hourly to characterize the immune response. The primary analytes of interest, leptin and fractalkine, were assayed via commercial radioimmunoassay and enzyme-linked immunosorbent assay kits, respectively. Exploratory analyses were performed on 20 secreted cytokine assays by multiplex cytokine panels, collected hourly. Exploratory analyses were also performed on testosterone, estrogen, and cortisol levels, collected hourly. Additionally, standard clinical complete blood counts with differential (CBC-D) were collected before LPS administration and at the end of the session. The fibromyalgia group demonstrated enhanced leptin and suppressed fractalkine responses to LPS administration. In the exploratory analyses, the fibromyalgia group showed a lower release of IFN-γ, CXCL10, IL-17A, and IL-12 and higher release of IL-15, TARC, MDC, and eotaxin than the healthy group. The results of this study suggest that fibromyalgia may involve an altered immune response to TLR4 activation.

1. Introduction

Fibromyalgia (FM) is a chronic multi-symptom pain disorder with a population prevalence of approximately 5% (Jones et al., 2015; Tu et al., 2020). Although the pathophysiological mechanisms of the condition have not been established, FM symptoms (e.g., hyperalgesia, fatigue, malaise, and cognitive disruption) resemble the classic sickness response, including symptoms such as elevated heart rate and general malaise (Kelley et al., 2003; Wolfe et al., 2010). Historically, observational studies have not consistently shown a significantly altered inflammatory biomarker profile in FM patients (Rodriguez-Pintó et al., 2014). However, some studies have identified elevated proinflammatory cytokines in FM, particularly, TNF-α (O'Mahony et al., 2021), IL-6 (Rodriguez-Pintó et al., 2014; O'Mahony et al., 2021), and CXCL8 (Rodriguez-Pintó et al., 2014; Kadetoff et al., 2012; Bains et al., 2023). Studies have found increased levels of these in the plasma of FM patients, and there appears to be a correlation with the severity of clinical symptoms (Rodriguez-Pintó et al., 2014). Although these cytokines are not considered diagnostic biomarkers, these findings do support the presence of an underlying inflammatory mechanism in FM.

Numerous factors have been shown to influence FM symptoms, including psychological stress, physical exertion, weather changes, impaired sleep, and hormonal fluctuations (Gomez-Arguelles et al., 2022; Vincent et al., 2016; Schertzinger et al., 2018). The intricate relationship between these triggers and symptoms involves a multifaceted interplay of diverse pathophysiological factors, including aberrant

* Corresponding author. Neuroinflammation, Pain, & Fatigue Laboratory, Department of Psychology, University of Alabama at Birmingham, 1300 University Blvd, Birmingham, AL, 35294, USA.
E-mail addresses: chloej@uab.edu (C. Jones), youngerlab@uab.edu (J. Younger).
[1] These two authors contributed equally to this work.

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central nervous system processing, neurotransmitter alterations, dysregulated pain responses, and immune disturbances. Notably, Toll-like receptor 4 (TLR4) has emerged as a particularly critical component of the innate immune system in chronic pain (Lacagnina et al., 2018), thereby warranting further investigation as a potential target for FM. Supporting the hypothesis of sensitized immune responses mediated by TLR4 in FM are two main lines of research. First, *in vivo* TLR4 activation through lipopolysaccharide (LPS) administration replicates core FM symptoms, including increased somatic and visceral pain responses (Hutchinson et al., 2013; Karshikoff et al., 2015; Wegner et al., 2014), fatigue (Lasselin et al., 2020), diminished motivation (Draper et al., 2018), reduced positive mood (Wegner et al., 2014; Kotulla et al., 2018; Kullmann et al., 2013), induction of sickness behavior (Jonsjö et al., 2020; Andreasson et al., 2019), and impaired cognitive performance (Reichenberg et al., 2001). Second, our previous work demonstrated that low-dose naltrexone, a TLR4 antagonist, reduces FM symptom severity (Younger et al., 2009, 2013; Parkitny et al., 2017). Based on these findings, we theorize that (1) FM involves a pathophysiologically-sensitized immune response to minor immune insults, which could explain the periods of symptom exacerbation (commonly referred to as FM flares) experienced by patients, and (2) FM involves exaggerated immune responses to certain stimuli which necessitate a specific provocation for their quantifiable manifestation. Despite dysregulated TLR4 activity being implicated in several chronic pain conditions (Grace et al., 2014; Nicotra et al., 2012; Kosek et al., 2016; Albrecht et al., 2018), we are not aware of any study that has experimentally tested *in vivo* innate immune responses for any chronic pain condition, including FM. We aimed to determine if FM involves abnormal innate immune responses to minor immune challenges using experimental intravenous low-dose LPS administration. LPS was administered to eight women with FM and eight healthy women at very low (0.1 ng/kg) and low (0.4 ng/kg) dosages. Cytokines, chemokines, and acute-phase proteins were measured at baseline, then hourly after LPS administration.

Two analytes of interest were selected and quantified with specialized immunoassays. The first analyte was the adipokine leptin which has been shown to induce TLR4 expression via the JAK2-STAT3 pathway in rodents (Jiang et al., 2021). In women, higher levels of leptin have been associated with lower preoperative pain thresholds, higher postoperative analgesic use (Akkececi et al., 2019), and greater self-reported pain (Younger et al., 2016). Higher baseline levels of leptin have been reported in patients with FM (Homann et al., 2013, 2014; Fietta et al., 2006; Ataoglu et al., 2018; Koca et al., 2020). However, some studies reported lower or unchanged leptin in FM (Paiva et al., 2017; Ablin et al., 2012; Olama et al., 2013). Abnormal leptin secretion in FM may involve a contextual dynamic response that requires an experimental provocation to reliably distinguish FM from healthy individuals. We thus hypothesized that individuals with FM would show greater increases in leptin in response to LPS administration compared to healthy controls.

The second analyte of interest was fractalkine (CX3CL1). CX3CL1 is a chemokine that exerts its effects through its sole receptor CX3CR1 which is predominantly expressed on microglia centrally and monocytes/macrophages peripherally. CX3CL1 has been shown to attenuate proinflammatory responses of reactive microglia in multiple models of inflammation, including neurotoxicity (Cardona et al., 2006; Lauro et al., 2015), cerebral ischemia (Lauro et al., 2019; Cipriani et al., 2011), experimental autoimmune encephalomyelitis, (Garcia et al., 2013) and LPS-mediated inflammation (Corona et al., 2010; Inoue et al., 2021; Mattison et al., 2013). In contrast, fractalkine (CX3CL1) acts as a pro-inflammatory chemokine in the case of macrophages and other systemic immune cells (Nanki et al., 2017). These divergent effects can likely be attributed to unique environmental and cell characteristics which likely contribute to the functional differences observed between these two cell types. Based on the promising evidence from multiple studies suggesting the involvement of fractalkine signaling in chronic pain, we formulated a hypothesis that individuals with FM would exhibit an altered fractalkine response upon the administration of lipopolysaccharide (LPS) (D'Haese et al., 2010). This hypothesis was driven by the notion that such alterations in fractalkine response may reflect changes in immune responses associated with FM.

Because LPS has never been administered to FM patients, we also characterized the immune response by quantifying a range of cytokines, chemokines, and acute-phase proteins using individual and multiplex assays. Lastly, a complete blood count with differential was conducted to assess for clinically-evident FM abnormalities in immune cell responses. No specific hypotheses were tested for these blood tests.

2. Materials and methods

2.1. Study participants

The study protocol was approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB) and by a US Federal Drug Administration Investigational New Drug Approval (IND-16396). Women aged 18–55 with a clinical diagnosis of FM, and a group of healthy women without pain were recruited. Participants were recruited via an in-house database of community individuals who had indicated an interest in research participation. Participants were additionally recruited via posted advertisements.

Inclusion criteria for all participants included: body mass index (BMI) of 18–39 to minimize the potential confounding effects of obesity on inflammation (Schmidt et al., 2015), normal cardiovascular function (resting heart rate >55 bpm; supine systolic blood pressure 100–140 mmHg and diastolic 60–90 mmHg), normal 12-lead electrocardiogram (ECG) with QTc interval <450 msec and QRS interval <120 msec, erythrocyte sedimentation rate <60 mmHr, C-reactive protein <3 mg/L, negative rheumatoid factor, antinuclear antibodies <1:60, and depression subscale score of <13 on the Hospital Anxiety and Depression Scale (Zigmond et al., 1983). All FM participants were required to meet the 2010 American College of Rheumatology criteria for a diagnosis of primary FM and report an average daily pain level >4 out of 10 on a standard pain visual analog scale.

Exclusion criteria for all participants included: a documented history of rheumatologic or autoimmune disease, current infection, current use of anti-inflammatory or immunomodulatory drugs, acute illness or fever within the last month, surgical procedures in the preceding three months, current or previous chronic use of opioids medication, current pregnancy, self-reported use of any drugs of abuse, nicotine use in the past 12 months, alcohol intake greater than an average of one drink per day, non-steroidal anti-inflammatory drug use for the past week, vaccines in the past 4 weeks, or antibiotics in the past 3 months.

2.2. Experimental design

Participants completed an initial phone screening with an investigator and an in-person screening interview and examination at the UAB Clinical Research Unit to determine eligibility. Consent was obtained from all participants at the time of screening. The following were collected: participant's medical history, Fibromyalgia Assessment Form (Wolfe et al., 2016), Fibromyalgia Impact Questionnaire (Burckhardt et al., 1991), Brief Pain Inventory (Cleeland et al., 1994), short form of the Depression Anxiety and Stress Scales [DASS-21] (Parkitny et al., 2010), 12-lead ECG, and a blood sample (complete blood count, erythrocyte sedimentation rate, thyroid stimulating hormone, free thyroxine, C-reactive protein, antinuclear antibodies, rheumatoid factor, liver function tests, and human chorionic gonadotropin (hCG)).

The two experimental sessions were held between two and four weeks apart to mitigate potential carry-over effects from LPS-mediated immune activation. The sessions commenced at 07:30–08:30 a.m. to control for possible diurnal variation in immune responses (Scheiermann et al., 2013). At the beginning of each session, inclusion criteria

were reassessed via an interview and clinical examination that included body weight and height, vital signs, a 12-lead ECG, and a urine pregnancy test (human chorionic gonadotropin; hCG). Bilateral antecubital intravenous access was secured and left *in situ* for the duration of each session. In all instances, one intravenous cannula was used to deliver the LPS and the contralateral cannula was used to obtain blood samples. Throughout all sessions, vital signs and the ECG were monitored for safety, with study cessation determined by: emergent mean arterial pressure outside the 70–110 mmHg range, bradycardia (<50 bpm) or tachycardia (>100bpm) at rest, ECG abnormalities, sinoatrial arrest, or physician or nursing staff concern about the patient's wellbeing. Additional data were collected concomitant with each blood draw, primarily to assist with patient monitoring. These included: blood glucose levels using an Accu-Chek Inform II system (Roche Diagnostics, USA), blood pressure, heart rate, oxygen saturation, respiration rate, and body temperature.

The LPS infusion was prepared on-site immediately before administration, following manufacturer protocols for reconstitution and dilution under sterile conditions. In short, the product was reconstituted and initially diluted with sterile water, then diluted to the final dose in 30 mL sterile saline within 15 min of administration. For each participant, a single fresh vial of Clinical Center Reference lyophilized endotoxin (lipopolysaccharide/LPS; lot 94332B1) was used, prepared from *Escherichia coli* O113:H10:K by List Biological Laboratories, Inc. The infusion was administered at a slow rate of 1 mL/min over 30 min using an infusion pump (Taudorf et al., 2007). In the first session, LPS was administered at a very low dosage of 0.1 ng/kg of participant body weight, and in the second session at a low dosage of 0.4 ng/kg. The selection of these doses was guided by the following considerations. First, previous research demonstrated that intravenous administration of endotoxin at 0.4 ng/kg significantly amplified the response to capsaicin in healthy adults, leading to increased allodynia, hyperalgesia, and flare (Hutchinson et al., 2013). Second, based on our hypothesis of an exaggerated immune response to TLR4 activation in FM, we postulated that this specific patient population would exhibit heightened immune responses even at extremely low doses.

The first blood draw for baseline assessments occurred immediately before the start of the LPS infusion. Blood draws were conducted hourly, to obtain a maximum of 9 blood draws. This specific time frame was chosen to capture the immediate immune cytokine expression in response to endotoxin. Although the release kinetics of cytokines can vary depending on the specific cytokine, it is generally observed that peak concentrations are reached within the first 2–4 hours following endotoxin administration (Kiers et al., 2017). For each blood draw, a waste tube was first drawn, and the intravenous cannula was cleared with sterile saline following the draw. Blood samples were immediately chilled on wet ice, promptly processed by centrifugation to extract the plasma component, aliquoted, and immediately stored at −80 °C until analysis (<12 months storage time). Samples for clinical assessments (e.g., complete blood count with differential; CBC-D) were immediately sent to UAB Clinical Laboratories for testing. Individuals were initially rested in bed in a supine position to permit appropriate clinical monitoring and were allowed to freely move about the room starting 2 hours after infusion. A meal of approximately 400 kcal was provided to each participant 2.5 hours after the infusion. The meal was standardized to caloric quantity, macronutrients, and timing, to offset any potential effects of food intake on the immune response, as well as naturally fluctuating pre- and post-prandial leptin levels (Karandish et al., 2012). A second meal was given 6.5 hours after infusion. Water was encouraged and provided *ad libitum*. The schedule for the experimental protocol can be seen in Fig. 1. Participants were compensated USD 25 for attending the screening session and USD 500 for each of the experimental sessions for a total of USD 1025.

2.3. Primary outcome variables

Assays for the primary outcome variables (leptin and fractalkine) were conducted by the UAB Human Metabolism/Physiology Core. Leptin was quantified with commercial Meso Scale Discovery (MSD, Rockville, MD) human leptin kits according to manufacturer protocols. Leptin assays were performed in singlet samples. Fractalkine was quantified with commercial R&D Systems human CX3CL1/fractalkine Quantikine ELISA kits according to manufacturer protocols. Assays were performed in duplicate. The inter- and intra-assay coefficients of variation (CVs) were determined and are shown in Table 1 and Table S1 in supplementary materials.

2.4. Exploratory outcome variables

Assays for the exploratory outcome variables were conducted at the UAB Human Metabolism/Physiology Core. Analytes were quantified with an MSD (Rockville, MD) V-plex Human Cytokine 30-Plex Panel kit (Proinflammatory Panel 1 [Human], Chemokine Panel 1 [Human], and Cytokine Panel 1 [Human]). Assays were performed in singlet samples according to manufacturer protocols. The following analytes were quantified with the V-plex Human Cytokine 30-Plex Panel kit: CCL2/MCP-1, CCL4/MIP-1β, CCL11, CCL13/MCP-4, CCL22/MDC, CCL26, CCL3/MIP-1α, CXCL10/IP-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-12p70, IL-13, IL-15, IL-16, IL-17A, interferon-gamma (IFN-γ), thymus and activation-related chemokine (CCL17/TARC), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-beta (TNF-β), and vascular endothelial growth factor (VEGF). C-reactive protein (CRP) and serum amyloid A (SAA) were measured with MSD (Rockville, MD) Vascular Injury Panel 2 [Human] kits according to manufacturer protocols. Testosterone, estrogen, progesterone, and cortisol were quantified using a Tosoh Bioscience Automated Immunoassay Analyzer 900 (South San Francisco, CA).

CBC-D, including CRP quantification, were conducted at the UAB Hospital Clinical Laboratories. The CBC-D tests were conducted at baseline to test for general health and thus to ensure participant safety directly prior to the LPS infusion. CBC-D tests were re-assessed 8 hours following infusion prior to releasing participants from the Clinical Research Unit. Counts and automated differentials were conducted

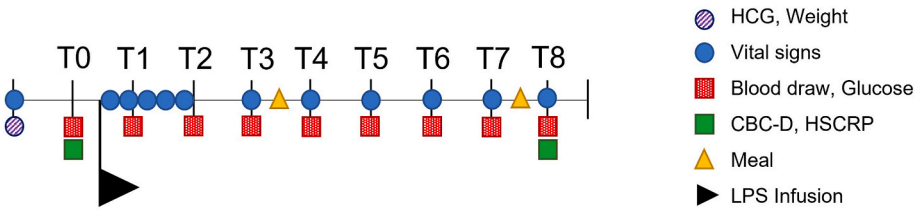

Fig. 1. Experimental protocol for 0.1 ng/kg and 0.4 ng/kg sessions.
Note. HCG = human chorionic gonadotropin urine pregnancy test; Vital signs = blood pressure, heart rate, oxygen saturation, respiration rate, body temperature, and electrocardiogram; CBC-D = complete blood count with differentials; HSCRP = high-sensitivity C-reactive protein; LPS = lipopolysaccharide. All sessions commenced between 7:30–8:30am to control for diurnal variation in immune responses. T0–T8 represent time points in hours relative to the LPS infusion. LPS was administered at a slow rate of 1 mL/min over 30 min. HSCRP and CBC-D were collected at baseline and 8 hours post-LPS infusion.

Table 1
Analytes included in analyses.

Analyte	Detectable Values[a]	Minimum Sensitivity	Unit	Intra-assay CV%	Inter-assay CV %
Leptin	99.306%	0.242	ng/ml	6.79	1.37
Fractalkine/ CX3CL1	99.306%	0.20	ng/ml	3.53	6.55
CRP	98.958%	0.00305	mg/L	2.1	10.6
SAA	99.306%	13.5	ng/ml	3.5	10.3
CCL2/MCP-1	99.653%	0.31	pg/ml	5.46	4.15
CCL4/MIP-1β	99.653%	3.00	pg/ml	3.65	4.68
CCL11/ eotaxin	99.653%	5.44	pg/ml	10.14	11.30
CCL13/MCP-4	99.653%	4.76	pg/ml	4.81	9.75
CCL22/MDC	99.653%	4.60	pg/ml	4.82	7.05
CXCL10/IP-10	99.653%	1.1	pg/ml	5.5	5.9
IL-6	99.653%	0.023	pg/ml	8.120	14.150
IL-7	99.653%	0.34	pg/ml	2.71	11.81
IL-8	99.653%	0.100	pg/ml	5.100	4.190
IL-10	99.306%	0.061	pg/ml	6.750	12.320
IL-12	99.653%	0.78	pg/ml	3.85	3.97
IL-15	99.653%	0.27	pg/ml	6.27	7.27
IL-16	99.653%	7.2	pg/ml	9.6	18.2
IL-17A	99.653%	0.45	pg/ml	4.69	10.90
IFN- γ	93.750%	0.660	pg/ml	9.040	3.490
TARC	99.653%	2.30	pg/ml	7.05	10.54
TNF-α	99.653%	0.200	pg/ml	4.980	4.840
VEGF	99.653%	1.05	pg/ml	4.38	6.72
Testosterone	96.000%	10.00	ng/dl	10.21	3.63
Estrogen	93.667%	25.0	pg/ml	4.1	4.1
Cortisol	100.000%	0.20	ug/dl	5.19	1.66

[a] Percentage of sample that contained detectable values for each analyte.

using the Coulter DxH 800 Analyzer based on manufacturer protocols. High-sensitivity CRP (hs-CRP) was also obtained directly prior to LPS infusion and 8 hours following infusion for participant safety. The hs-CRP was obtained using the Beckman Coulter AU System Analyzer at the UAB Hospital Clinical Laboratories based on manufacturer protocols.

2.5. Statistical analyses

Main analyses were conducted using linear mixed models in IBM SPSS v28 (Armonk, NY). For all models, the subject identifier was entered as the individual nesting variable, time (in hours) as the repeated measures index variable, and a compound symmetry repeated measures covariance structure was used. The models were constructed with three main effects: group (FM versus healthy), timepoint (entered as a continuous covariate predictor), and session (0.1 ng/kg versus 0.4 ng/kg). All two-way and three-way interactions were included in the model. A $p < 0.05$ statistical significance threshold was set for all analyses.

Repeated measures analysis of variance (ANOVA) with a between factor of group was used to evaluate changes in vitals and clinical blood tests pre- and post- LPS infusion for each session (T0, T8). A $p < 0.05$ statistical significance threshold was set for all analyses.

3. Results

3.1. Participants

A total of 41 individuals completed lab screening. Of these, 25 were excluded for abnormal lab results at the screening session. Excluded reasons included: QTc >450 msec (n = 6), abnormal CBC values (n = 10), inability to secure proper IV blood draws (n = 5), abnormal ECG patterns (n = 2), BMI out of bounds (n = 1), and elevated resting blood pressure (n = 1).

The final sample included eight women with FM and eight healthy control women (HC). The cohort baseline characteristics are shown in Table 2. The FM group was older ($t[14] = 3.26$, $p = 0.006$), and had a higher BMI ($t[14] = 2.90$, $p = 0.012$) compared to the HC. All individuals who started the experimental protocol completed both sessions. In the 0.4 ng/kg session, one individual exhibited highly elevated cytokine levels compared to the group (TARC, IL-16, MCP4) at baseline (7.14, 9.31, and 5.39 standard deviations above the mean, respectively). They also did not exhibit these unusually elevated TARC, IL-16, and MCP4 baseline values at the 0.1 ng/kg session (0.70, 0.37, and 0.95 standard deviations above the mean, respectively). Despite the absence of any observable technical errors in the assay or concurrent pathophysiological factors, a conservative approach was taken to not bias the analysis by excluding them from further consideration. As such, these participant's results were not used in the 0.4 ng/kg session analyses.

3.2. Vital signs

There were significant increases in heart rate ($p = 0.006$), temperature ($p = 0.001$), and blood glucose levels ($p < 0.001$) across both groups and dosages. There was a significant group × time interaction indicating that blood glucose levels rose higher in the healthy control group ($p = 0.046$). There were no significant group effects observed for heart rate, blood pressure, mean arterial pressure, temperature, respiration rate, or oxygen level.

3.3. Primary outcome variables

For fractalkine, the 3-way group × session × time interaction was not significant ($p = 0.917$), indicating that the group differences in the fractalkine time-courses were similar between the two sessions. There was a significant main effect for time ($p < 0.001$), with fractalkine values increasing during the session, and a significant main effect for session ($p < 0.001$), with higher levels observed in the 0.4 ng/kg session. The group × session interaction was significant ($p = 0.045$), with fractalkine increasing less in the FM group. The session × time interaction was also significant ($p = 0.042$), with the fractalkine increase being greater in the 0.4 ng/kg LPS session.

For leptin, the 3-way group × session × time interaction was not significant ($p = 0.735$), indicating that the group differences in the leptin time-courses were similar between the two sessions. The 2-way group × session interaction for leptin was significant ($p < 0.001$), with the FM group showing greater leptin levels in both sessions, and greater increases at the 0.4 ng/kg session. The results of statistical analyses for plasma leptin and fractalkine are presented in Fig. 2a and b.

3.4. Exploratory cytokine/chemokine analyses

Eleven analytes (CCL26, CCL3, GM-CSF, IL-1α, IL-1β, IL-2, IL-4, IL-5,

Table 2
Baseline participant characteristics.

	Group						
	Healthy (n = 8)		Fibromyalgia (n = 8)		Total (n = 16)		
	Mean	**SD**	**Mean**	**SD**	***t***	**df**	**p**
Age at Consent (years)	28.38	6.16	41.50	9.58	−3.26	14	**0.006**
Body Mass Index	23.58	2.78	29.55	5.11	−2.90	14	**0.012**
Self-Reported Pain (1–10)	0.00	0.00	5.69	1.28	−18.19	14	**< .001**
Baseline CRP (mg/L)[a]	1341.63	1372.61	1686.88	2206.93	−0.38	14	0.713
Baseline IL-6 (pg/mL)[a]	0.64	0.37	0.84	0.50	−0.89	14	0.389
Baseline TNF-α (pg/mL)	1.69	0.45	1.66	0.27	0.16	14	0.439
Baseline Heart Rate (bpm)	68.38	7.95	71.88	11.62	−0.70	14	0.493

[a] CRP, IL-6, and TNF-α baseline values are derived prior to infusion (T0) of the first experimental session.

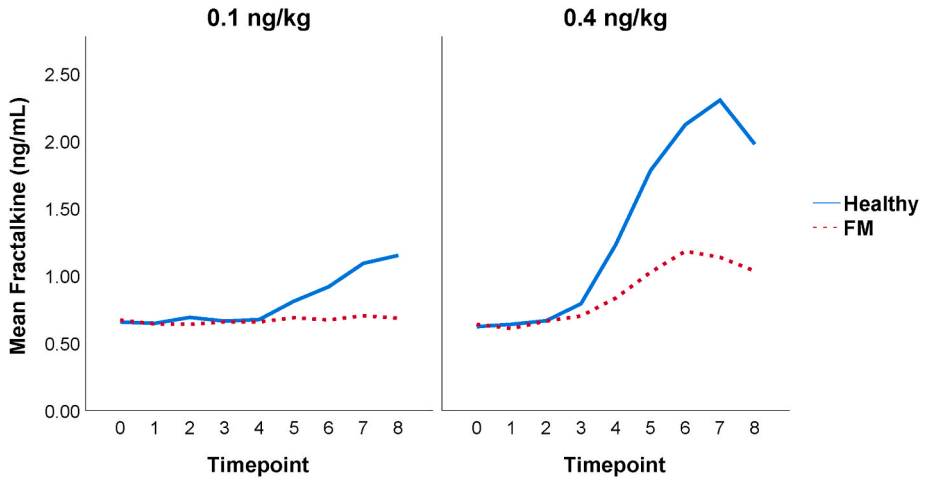

Fig. 2a. The time course of fractalkine following LPS administration.
Note. Women with FM show blunted fractalkine response to lipopolysaccharide. Lines are serum fractalkine (CX3CL1) concentrations in the healthy group (solid line) and FM group (dashed line). The left pane represents the 0.1 ng/kg LPS session, and the right pane is the 0.4 ng/kg LPS session. Timepoint is relative to completing the LPS infusion in hours.

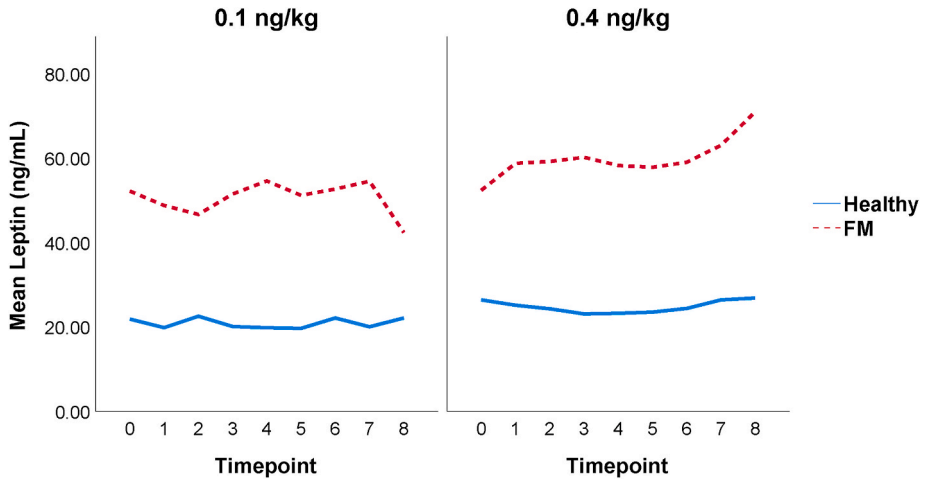

Fig. 2b. The time course of leptin following LPS administration.
Note. Women with FM show enhanced leptin response to lipopolysaccharide. Lines are leptin concentrations in the healthy group (solid line) and FM group (dashed line). The left pane represents the 0.1 ng/kg LPS session, and the right pane is the 0.4 ng/kg LPS session. Timepoint is relative to completing the LPS infusion in hours.

IL-12p70, IL-13, and TNF-β) were excluded from all analyses due to the high frequency of undetectable values. The tested analytes included: CRP, SAA, CCL2/MCP-1, CCL4/MIP-1β, CCL11/eotaxin, CCL13/MCP-4, CCL17/TARC, CCL22/MDC, CXCL10/IP-10, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17A, INF- γ, TNF-α, and VEGF. Results for the complete set of tested cytokines can be found in the supplemental results.

Several interleukin cytokines showed group differences. There was a significant group × session interaction for IL-15 ($p = 0.019$) with IL-15 increasing over time, and greater group differences emerging at the 0.4 ng/kg session. There was also a significant session × time interaction for IL-15 ($p < 0.001$), with levels increasing significantly more in the 0.4 ng/kg LPS session. IL-12 showed a significant main effect of time ($p <$

0.001) and main effect of group ($p = 0.007$), such that IL-12 increased over time in both groups but was lower overall in the FM group. Similarly, IL-17A was overall lower in the FM group ($p = 0.05$) but increased over time in both groups ($p < 0.001$). No other interleukin cytokines (IL-6, IL-7, IL-8, IL-10, or IL-16) showed significant group differences. A main effect of dosage, with no group differences, was found for IL-6 ($p = 0.029$) and IL-10 ($p = 0.007$), with levels higher in the 0.4 ng/kg LPS session.

Two analyses related to interferon showed group differences. First, IFN-γ release was observed to be suppressed in the FM group in both the 0.1 ng/kg and 0.4 ng/kg sessions (group × time $p < 0.001$). Second, CXCL10/IP-10, a chemokine that is released in response to IFN-γ, showed a significant group × time relationship ($p < 0.001$), and a significant session × time relationship ($p = 0.001$) with higher levels observed in the 0.4 ng/kg session.

Three additional analytes showed significant group differences. CCL17/TARC showed a significant group × session interaction ($p = 0.038$). In the 0.1 ng/kg LPS session, the FM group showed a greater peak in the release of CCL17/TARC ($p = 0.008$). Additionally, CCL22/MDC showed a significant group × time interaction ($p = 0.027$) with the FM group showing higher average MDC levels overall, and the healthy group showing a significant session × time interaction ($p = 0.012$). Finally, CCL11/Eotaxin was higher in the FM group in both sessions ($p = 0.032$).

For the acute phase protein SAA, there was a significant session × time interaction, with CRP increasing significantly more in the 0.4 ng/kg LPS session ($p < 0.001$), however there were no significant group differences. The other tested acute phase protein, CRP, showed no significant group differences, however, there was also a significant session × time interaction, with CRP increasing significantly more in the 0.4 ng/kg LPS session ($p = 0.01$).

CCL13/MCP-4 showed a significant session × time interaction ($p = 0.020$) without group effects. Steeper inclines were observed in MCP-4 seen during the 0.4 ng/kg session. There were no significant effects of group, dosage, or time on CCL2/MCP1.

No significant group effects were found for CCL4/MIP-1β, or VEGF. CCL4/MIP-1β was significantly increased in the 0.4 ng/kg LPS session in both groups ($p = 0.008$). VEGF increased significantly over time ($p < 0.001$), with no observed differences in group or session. All significant group differences and group interactions for the exploratory analytes can be seen in Table S2 of the supplementary materials. Significant group × time interactions for the exploratory analytes can be seen in Fig. 3a–c.

3.5. Exploratory hormone analyses

Progesterone was excluded from all analyses due to the high frequency of undetectable values. The tested hormones included testosterone, estrogen, and cortisol. There was a significant effect of dosage ($F[1, 247] = 10.295$, $p = 0.002$), and time ($F[1, 247] = 9.907$, $p = 0.002$) on testosterone, with a significant session × time interaction ($F[1, 247] = 11.665$), indicating that the time course of testosterone release was different between the two sessions. There was a significant effect of dosage on estrogen ($F[1, 247] = 14.076$, $p < 0.001$), with greater levels of estrogen observed in the 0.4 ng/kg session. Cortisol was significantly affected by dosage ($F[1, 264] = 7.400$, $p = 0.007$), and time ($F[1, 264] = 9.386$, $p = 0.002$). No significant group differences were observed for any of the tested hormones. The results for the hormone analyses can be seen in Table 3.

3.6. Clinical tests

There were no missing data for any of the clinical blood tests. CBC-D values were obtained at baseline, before LPS administration, and at hour 8 of the session (T8). Following 0.1 ng/kg LPS administration, there were no significant group differences in any of the clinical tests. However, we observed a non-significant trend for the group × time interaction for WBCs. In the 0.4 ng/kg dose LPS administration session, this difference was significant ($F[1, 14] = 5.272$, $p = 0.038$). Post hoc tests revealed a significant increase in WBCs in both sessions for the HC ($F[1, 14] = 39.089$, $p = 0.001$ and $F[1, 14] = 6.195$, $p = 0.042$) and the FM group ($F[1, 14] = 35.640$, $p = 0.001$ and $F[1, 14] = 32.372$, $p = 0.001$), with the FM group showing a greater average increase. Additionally, in the 0.4 ng/kg session, we observed a significant interaction in blood glucose levels ($F[1, 14] = 5.272$, $p = 0.038$). Post hoc tests indicated a significant increase in blood glucose levels in both sessions for the HC group ($F[1, 14] = 7.567$, $p = 0.033$ and $F[1, 14] = 31.177$, $p = 0.001$), while no significant change was observed in the FM group ($F[1, 14] = 4.795$, $p = 0.065$ and $F[1, 14] = 4.795$, $p = 0.065$). These findings suggest that both HC and FM individuals exhibit signs of an immune response to 0.1 and 0.4 ng/kg doses of LPS, but FM may be associated with a slightly increased WBC response and a reduced glucose response. The results for the clinical blood tests can be seen in Table 4.

3.7. Additional analyses of potential confounders

Because the FM group was significantly older than the healthy control group (Table 2), additional analyses were conducted to mitigate the

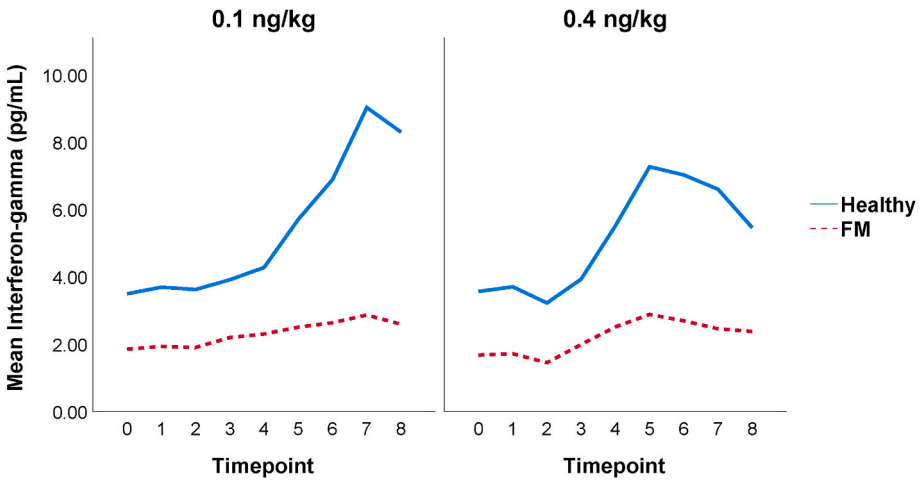

Fig. 3a. Time course of IFN-γ following LPS administration.
Note. Mean *IFN-γ* levels following LPS administration are shown. The healthy group is shown on the solid line and FM group is the dashed line. The left pane represents the 0.1 ng/kg LPS session, and the right pane is the 0.4 ng/kg LPS session. Timepoint is relative to completing the LPS infusion in hours.

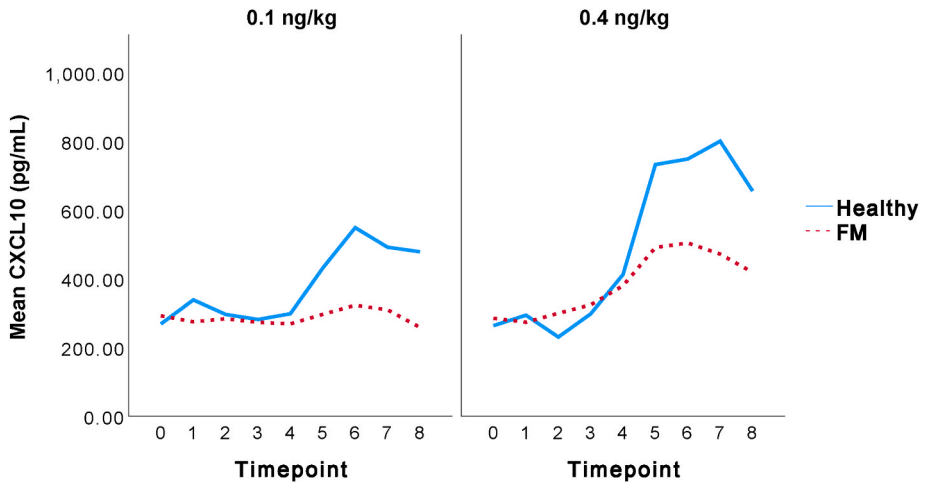

Fig. 3b. Time course of CXCL10 following LPS administration.
Note. Mean CXCL10 levels following LPS administration are shown. The healthy group is shown on the solid line and FM group is the dashed line. The left pane represents the 0.1 ng/kg LPS session, and the right pane is the 0.4 ng/kg LPS session. Timepoint is relative to completing the LPS infusion in hours.

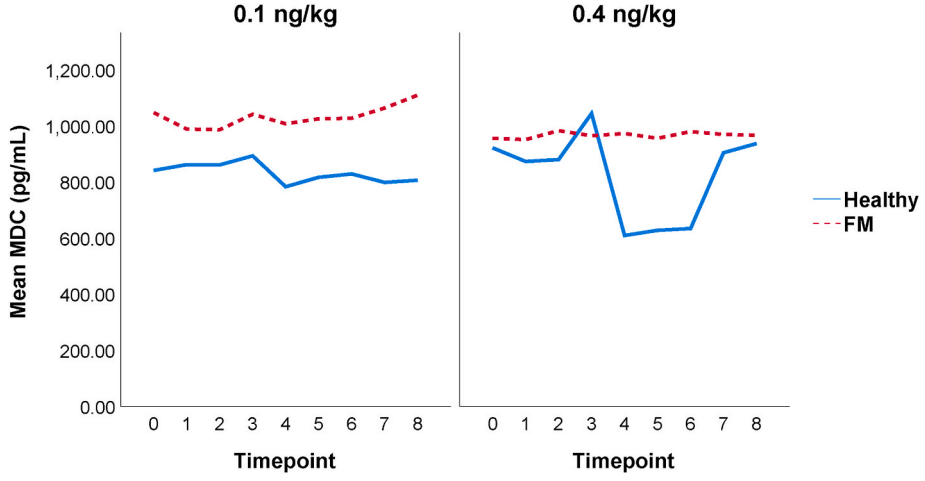

Fig. 3c. Time course of MDC following LPS administration.
Note. Mean MDC levels following LPS administration are shown. The healthy group is shown on the solid line and FM group is the dashed line. The left pane represents the 0.1 ng/kg LPS session, and the right pane is the 0.4 ng/kg LPS session. Timepoint is relative to completing the LPS infusion in hours.

Table 3
Hormone analyses.

	Group	Mean	Group	Session	Time	Time × Session
			F	F	F	F
Testosterone	HC	33.445	3.289	**10.295****	**9.907****	**11.665*****
	FM	19.957				
Estrogen	HC	106.368	0.851	**14.076*****	1.596	0.671
	FM	79.029				
Cortisol	HC	10.126	2.456	**7.400****	**9.386****	0.384
	FM	8.003				

**p < 0.01. ***p < 0.001.

potential contribution of age to the primary and exploratory outcome results. As age did not vary at the within-subject level, rather than including it as a covariate in the model, we performed a sensitivity analysis by truncating the data to remove age differences between groups. The two oldest participants from the FM group and the two youngest from the healthy control group were removed from the dataset (n = 12). The remaining sample showed no significant difference in age ($t[10] = 1.90$, p = 0.09). All analyses were rerun on the truncated sample. All significant results previously reported remained significant.

The FM group also had a significantly higher BMI than the healthy control group. Because BMI is a known factor in leptin levels, we also conducted a sensitivity analysis of leptin by utilizing a truncated dataset. The two participants with the highest BMI from the FM group and the two participants with the lowest BMI from the healthy group were removed from the analyses. This truncated sample showed no significant difference in BMI ($t[9] = 1.00$, p = 0.34). Analyses were performed as

Table 4
Clinical blood tests by session.

	Group	Session 1				Session 2			
		Pre	Post	Group Effect		Pre	Post	Group Effect	
		Mean	Mean	F	Sig.	Mean	Mean	F	Sig.
MAP	HC	82.6	83.6	2.373	0.147	83.0	82.1	0.171	0.686
	FM	86.3	82.5			80.1	80.4		
Heart rate	HC	68.0	76.6	0.829	0.379	67.8	78.6	0.053	0.822
	FM	71.9	74.5			70.0	80.0		
Temperature	HC	98.2	98.3	0.336	0.572	98.3	98.8	1.874	0.193
	FM	98.2	98.4			98.3	98.5		
Resp. rate	HC	17.6	17.1	0.739	0.406	17.0	17.8	0.19	0.893
	FM	16.3	17.0			16.9	17.8		
SpO_2	HC	98.9	99.4	2.349	0.149	99.0	98.9	0.255	0.622
	FM	99.1	98.6			98.8	98.9		
Blood Glucose	HC	87.0	119.0	1.010	0.333	**80.6**	**130.9**	**10.105**	**0.007**
	FM	89.4	107.3			**95.9**	**110.5**		
ESR (mm/hr)	HC	99.1	98.6	0.187	0.673	2.4	3.3	0.007	0.932
	FM	11.6	11.3			9.6	10.6		
Hemoglobin	HC	13.2	12.9	0.017	0.897	12.8	12.7	0.026	0.874
	FM	12.6	12.4			12.0	11.9		
Hematocrit	HC	37.7	37.6	0.494	0.494	37.4	36.9	0.063	0.805
	FM	37.0	36.4			35.1	34.8		
WBC	HC	6.1	7.7	3.998	0.067	**6.4**	**7.6**	**5.272**	**0.038**
	FM	6.0	8.7			**6.1**	**8.9**		
Red Blood Cells	HC	6.1	7.7	3.998	0.067	4.2	4.2	0.004	0.952
	FM	6.0	8.7			4.0	4.0		
MCV	HC	89.3	89.9	4.533	0.053	89.4	89.0	0.720	0.411
	FM	88.6	88.3			88.0	84.5		
MCH	HC	31.0	31.0	0.156	0.700	30.5	30.5	0	–
	FM	30.1	30.3			29.9	30.2		
MCHC	HC	34.7	34.3	3.241	0.095	34.3	34.3	0.568	0.464
	FM	33.9	34.0			33.9	33.5		
Platelets	HC	217.9	202.1	2.785	0.119	235.0	213.5	4.535	0.051
	FM	226.2	224.8			226.0	215.6		
RDW	HC	13.2	13.0	0.831	0.379	12.9	13.0	0.206	0.657
	FM	13.3	13.2			13.4	13.4		
MPV	HC	8.9	8.9	0.867	0.369	8.8	8.8	2.333	0.149
	FM	8.3	8.1			8.1	8.4		
Neutrophils	HC	60.1	71.1	1.243	0.285	63.1	73.5	1.067	0.319
	FM	59.1	65.9			57.1	71.8		
Absolute Neutrophils	HC	3.7	5.5	0.649	0.435	4.1	5.6	4.533	0.051
	FM	3.6	5.7			3.5	6.3		
Lymphocytes	HC	29.4	20.4	1.389	0.260	27.4	18.5	0.011	0.917
	FM	31.3	26.0			29.3	20.8		
Monocytes	HC	8.1	6.6	0.96	0.762	6.6	6.5	2.947	0.108
	FM	7.4	5.5			7.4	5.8		
Eosinophils	HC	2.1	1.3	2.814	0.117	1.9	1.1	0.84	0.776
	FM	2.1	1.9			2.1	1.3		
Basophils	HC	0.6	0.4	0.903	0.359	0.6	0.5	1.615	0.224
	FM	0.9	0.4			0.8	0.3		
hs-**CRP**	HC	1.3	1.7	0.166	0.690	2.2	3.1	2.690	0.125
	FM	1.3	1.9			1.7	3.4		

Note. Significant group differences are displayed for the clinical blood tests and vital measurements, by session. Pre- and post- LPS infusion means are displayed for each group. Analyses were conducted using repeated measures ANOVAs. Significant group differences of $p < 0.05$ are bolded. No group differences were significant using a False Discovery Rate of 0.10.

previously, and the group × time interaction for leptin remained significant ($p = 0.02$), suggesting that results regarding leptin were robust to group differences in BMI.

4. Discussion

In this pilot study, we tested the innate immune response of women with FM, and healthy controls, following low-dose LPS administration. The FM group showed an abnormal response to LPS in several immune parameters. In general, these findings align with *in vitro* studies that

have demonstrated heightened expression of proinflammatory cytokines in endotoxin-stimulated human whole blood and peripheral blood mononuclear cells obtained from women with pelvic pain (Evans et al., 2020; Schrepf et al., 2023). Additionally, a recent study conducted by our team revealed altered temperature and metabolic responses in response to *in vivo* endotoxin administration in women with FM (Mueller et al., 2023). Collectively, our current findings and these earlier observations suggest a potential link between abnormal immune responses, proinflammatory cytokines, and FM.

FM patients displayed a suppressed fractalkine/CX3CL1 response at both dosages of LPS. CX3CL1 is a chemokine that is constitutively expressed by immune and non-immune cells including neurons, as well as by microglia, astrocytes, and endothelial cells under pro-inflammatory stimulation conditions (Harrison et al., 1998; Lee et al., 2018; Yoshida et al., 2001); its expression is particularly pronounced in the central nervous system (Bazan et al., 1997). CX3CL1 is synthesized as a 95 kDa transmembrane form that is proteolytically cleavable into a smaller 70 kDa soluble signaling chemotactic protein (Chapman et al., 2000). In the brain, CX3CL1 constrains microglial activation and proinflammatory cytokine secretion (Lauro et al., 2019; Bachstetter et al., 2011; Mizuno et al., 2003).

Fractalkine has been shown to reduce the pro-inflammatory response of microglia. It has been reported to reduce microglia pro-inflammatory activity in response to brain ischemia (Lauro et al., 2019), and protect neurons in an amyotrophic lateral sclerosis model (Liu et al., 2019). Most directly relevant to the current study, fractalkine has been consistently found to attenuate pro-inflammatory microglial responses to LPS administration (Mizuno et al., 2003; Zujovic et al., 2000; Lyons et al., 2009), and is important in reducing the behavioral changes caused by TLR4 activation (Corona et al., 2010). It is also important to note that it is not known how well brain and serum fractalkine levels correlate. However, blood fractalkine has demonstrated utility as a potential biomarker for disease severity in neurological conditions such as ischemic stroke, Alzheimer's Disease, and Parkinson's Disease (Donohue et al., 2012; Gupta et al., 2022; Kim et al., 2008), suggesting that its peripheral levels reflect the underlying pathophysiology. However mechanistic studies are needed to identify the specific causal agents underlying our finding.

The FM group also showed a heightened leptin response to LPS administration. While leptin is principally known as an energy-regulating hormone associated with obesity (Flier, 1998), it has been more recently classified as a pro-inflammatory adipokine and reported to play important roles in systemic immune responses (Cava et al., 2004) and the sickness response (Harden et al., 2006). In particular, leptin has been shown to sensitize microglia and increase their secretion of proinflammatory factors (Lafrance et al., 2010). We have previously reported that tonic levels of leptin are associated with pain (Younger et al., 2016) and fatigue (Stringer et al., 2013) in women. Additionally, leptin levels have been reported to be elevated in FM, after controlling for BMI (Ataoglu et al., 2018; Koca et al., 2020) though some studies have reported lower leptin in FM (Paiva et al., 2017; Olama et al., 2013). The results of our study suggest that abnormal leptin levels in FM may be particularly pronounced after an immune trigger. Provocation studies such as ours may better distinguish FM from healthy groups than observational studies. The cumulative literature suggests that leptin should be further explored as a factor in the pain and fatigue associated with FM.

Most of the secondary cytokine multiplex analyses showed no differences between the FM and healthy control groups. Notable exceptions are group differences following LPS administration in IFN-γ, IP-10, IL-15, IL-12, IL-17A, eotaxin, TARC, and MDC levels. The FM group showed enhanced pro-inflammatory signaling of IL-15, TARC, MDC, and eotaxin contrasted with the healthy control group. However, in comparison to the healthy group, FM showed suppressed IFN- γ, IP10, IL-12, and IL-17A. Because of the exploratory nature of our multiplex assay and the untargeted nature of the tests performed, it is premature to interpret the significance of these apparent altered LPS responses in FM. If replicated, these results may indicate specific abnormalities in the FM immune response. In addition, subsequent to the administration of 0.4 ng/kg of endotoxin, we observed that individuals with FM demonstrated a more pronounced increase in WBCs. Additionally, the HC group, but not the FM group, showed a significant increase in blood glucose levels. It is important to note that our study was not designed to comprehensively assess changes in cell subtypes or metabolic responses related to innate immune activation. Nevertheless, the finding of potentially impaired glucose responses could potentially indicate abnormalities in immune, hormonal, glycogen storage, or other metabolic functions which should be further investigated in future studies.

Finally, to discern the potential triggers of heightened TLR4 activation in women with FM, we must carefully consider all potential contributing factors. Notably, some pelvic organ conditions involving chronic pain appear to be linked with dysregulated innate immunity at both local and systemic levels. For instance, the bacterial contamination hypothesis in endometriosis underscores the involvement of TLR4 activation in the pathological progression and symptomatic manifestation of this condition (Khan et al., 2018). Moreover, individuals with interstitial cystitis/bladder pain syndrome exhibit a positive correlation between their responsiveness to peripheral blood mononuclear cell (PBMC) stimulation by endotoxin and their symptom severity (Schrepf et al., 2014). Dysmenorrhea has been associated with an augmented proinflammatory chemokine load in the bloodstream (Roomruangwong et al., 2020) and has emerged as a discernible risk factor for the eventual onset of FM(Tu et al., 2020). Additionally, the hormonal fluctuations and concurrent sterile inflammation that occur during a typical menstrual cycle also exert marked immunomodulatory effects (Roomruangwong et al., 2020). While not an exhaustive list of pertinent evidence, these discoveries strongly imply that dysregulated innate immunity may constitute a prevalent characteristic in diverse pain syndromes. This emphasizes the importance of thorough screening for such conditions in research. Moreover, this underscores the need for a deeper understanding of the potentially intricate interplay between multiple coexisting pathophysiologies.

4.1. Limitations

There are several limitations and caveats to note regarding this preliminary study. Future research should consider several methodological changes when replicating these exploratory results. First, a notable limitation of the study is the absence of a placebo session. The target population primarily exhibits symptoms of pain and fatigue. Initially, this study aimed to test both doses of LPS, and therefore, a placebo control condition was not included. The main focus was to compare the FM group with the control group to detect significant deviations from the normal condition. However, it is crucial to acknowledge without a true placebo, it is possible that diurnal cycle effects may be influencing some of the identified changes, rather than solely reflecting a genuine response to LPS. While we acknowledge the limitations imposed by the lack of a placebo control, we believe that our study design provides valuable insights into the differential responses of the FM group compared to the control group, allowing us to observe significant departures from normal conditions. Additionally, the observed dose-response relationship between the two LPS doses further strengthens the validity of our findings.

Secondly, the sample size is limited and prevents generalizations to the larger FM population. This proof-of-concept study suggests that further exploration of immune responses in FM are warranted, and hints at the pathophysiological mechanisms of the condition.

A third limitation is that leptin and the exploratory analytes assays were performed in singlet due to limited resources, while fractalkine was able to be performed in duplicate. To address the potential loss of precision associated with the quantification of singlet samples, we rigorously monitored expression patterns within each participant throughout

the entire time course of each session. When any potential outliers that might have influenced the accuracy of the results were identified, the relevant samples were subjected to re-analysis, ensuring the validation and reliability of our findings. However, duplicate or triplicate quantification for all analytes of interest is strongly recommended as best practice for any future research.

Fourth, a significant limitation is that the groups were not perfectly age-matched or BMI-matched. The FM group was older than the healthy controls. To address this limitation, we performed sensitivity analyses which corroborated all our main findings, suggesting that these results were largely robust to the group difference in age.

Although each LPS infusion was individualized to each participant's weight, based on leptin's established relationship to BMI, group differences in BMI could have driven the significant group results. Therefore, we again performed a sensitivity analysis and found that the main leptin interaction remained significant.

Fifth, the LPS dosages used were atypically low for human endotoxemia studies. These doses were selected to stimulate the innate immune system while avoiding a marked infection-like response. We note that group differences were clearer at the 0.4 ng/kg dosage. It is possible that true group differences did not emerge because of the low dosage used. More typical LPS dosages such as 0.8 ng/kg may have revealed additional group differences.

Sixth, because the 0.1 ng/kg dosage always preceded the 0.4 ng/kg, it is possible that responses in the larger dose were primed by the first session. As part of our clinician safety protocol, the lower dose of the treatment was consistently administered first in order to mitigate any potential unexpected adverse reactions. This approach was chosen to account for the possibility of a primed immune system in the study population, hypothesizing that severe and/or prolonged symptoms could occur. Given the lack of prior testing of such immune responses in this specific population, this cautious sequential dosing strategy aimed to ensure participant safety and to monitor the initial impact of the treatment before proceeding to higher doses. Even with $\geq$2 weeks of separation between the two sessions, carryover effects are possible, including priming or tolerance effects which may suggest additional pathophysiological mechanisms and should be tested in subsequent studies.

Seventh, the study only included women with FM. Recent studies have demonstrated that chronic pain immune mechanisms are sex divergent and may involve different immune cells (Mapplebeck et al., 2017) and thus potentially immune mechanisms. As such, subsequent work will need to closely investigate both sexes. However, conducting a study exclusively utilizing women in the investigation of primary FM is justified due to the markedly higher prevalence of the condition in females compared to males (Marques et al., 2017). Particularly given the study sample size, our approach allows for a more focused examination of the specific disease manifestations, underlying mechanisms, and potential sex-specific pathophysiological factors in the population where it is most commonly diagnosed.

Finally, subsequent work will need to investigate the influence of sex hormones and their potential interactions with immune responses, particularly given that menstrual cycle hormone fluctuations may be implicated in FM symptomatology (Schertzinger et al., 2018). Future studies should consider examining this variable, although it is important to note that accurate cycle classification requires sophisticated approaches such as hormone quantification and/or ultrasonography (Allen et al., 2016).

4.2. Conclusion

We found that individuals with FM have an altered immune response to low-level TLR4 activation by LPS. The observed abnormalities did not involve most of the classic innate immune response mediators, which were found to be similar between groups. Rather, the lesser assessed analytes such as leptin and fractalkine may be involved in the pathophysiology of FM. We observed that, in response to the low-level immune challenge, individuals with FM exhibited heightened pro-inflammatory responses and suppressed anti-inflammatory responses, compared to the healthy control group. These variations in immune response could potentially be linked to the differential expression of leptin and fractalkine, although the exact underlying mechanisms are expected to be complex and multifaceted. One plausible explanation is that leptin and fractalkine may play a role in modulating immune responses to immune challenges. However, further investigation through larger and more focused studies is necessary to thoroughly test these hypotheses and develop a deeper understanding of the involvement of leptin and fractalkine in the altered immune responses observed in FM in this study.

These findings are consistent with the view that FM may involve abnormally large or extended inflammatory reactions to daily immune insults. Partially supporting this hypothesis is our previous work showing that the TLR4 antagonist naltrexone can reduce pain and proinflammatory cytokine production in FM (Younger et al., 2009, 2013; Parkitny et al., 2017). These findings are further corroborated by positron emission tomography (PET) studies that have observed elevated uptake of the translocator protein ligand [^{11}C]PBR28, indicating glial activation. In contrast, the uptake of the monoamine oxidase-B ligand [^{11}C]-L-deprenyl-D_2, which primarily reflects astrogliosis, did not show significant alterations. These PET findings provide additional support for the involvement of glial activation, particularly microglia, in the pathophysiology of FM. (Albrecht et al., 2019). Further exploration of microglia and astrocyte activation, and abnormal systemic immune responses to triggers, is warranted.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The authors of the study thank the individuals who participated in the study. We also acknowledge the staff of the UAB Clinical Research Unit for their assistance in data collection. We also thank Dr. Timothy Ness for participant screening and monitoring participant safety. Original data will be made available to researchers upon request and receipt of a data sharing agreement. Data analysis code will be shared freely upon request. The authors declare that they have no conflicts of interest relevant to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100707.

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