



Modulatory role of prenatal alcohol exposure and adolescent stress on the response to arthritis challenge in adult female rats

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Summary

Background There are known environmental risk factors associated with rheumatoid arthritis; however, less is known regarding how the prenatal environment impacts later-life risk for rheumatoid arthritis. Based on preliminary clinical data suggesting that individuals with fetal alcohol spectrum disorder (FASD) are at higher risk for autoimmune disorders, this study investigated the modulatory impact of prenatal alcohol exposure (PAE) on the inflammatory disease profile in an adjuvant-induced arthritis rat model.

Methods Pregnant rats received liquid ethanol or control diet throughout gestation. To model the increased exposure to stressors often experienced by individuals with FASD, adolescent offspring were exposed to chronic mild stress (CMS) or remained undisturbed. In adulthood, experimental arthritis was initiated and rats terminated either at the peak or following resolution from inflammation to assess endocrine, immune, and histopathological outcomes.

Findings PAE rats had an increased incidence and severity of, and impaired recovery from, arthritis. Increased joint damage was observed in PAE animals, even in the face of apparent recovery from the clinical signs of arthritis, while it appeared that oestradiol may have a protective role. Moreover, with the combination of PAE and adolescent stress, increased macrophage density was detected in the synovium of PAE but not control rats.

Interpretation These findings demonstrate that PAE alters the severity and course of arthritis, highlighting the potential immunomodulatory impact of adverse prenatal exposures. In particular, these data have implications for understanding preliminary data that suggest a heightened propensity for autoimmune disorders in individuals with FASD.

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Introduction

Rheumatoid arthritis, the chronic inflammatory disease of the articulating joints, has a global prevalence of approximately 0.24%.¹ However, the prevalence of rheumatoid arthritis is much higher in certain subgroups – as high as 4–8% in certain North American Indigenous populations² and approximately two times higher in women than men.¹ In addition, while genetic vulnerabilities have been identified for rheumatoid arthritis, and there is a growing list of known

environmental risk factors and triggers of rheumatoid arthritis, such as smoking, obesity, and recent infection,^{3,4} it is less clear whether the prenatal environment can impact later life risk of autoimmune disorders such as rheumatoid arthritis.

The present study utilized our well-established animal model of prenatal alcohol exposure (PAE) to investigate the impact of this prenatal environmental insult on the incidence, severity, and course of adjuvant-induced arthritis using an outbred rat strain (Sprague-Dawley). This PAE animal model is used to study fetal alcohol spectrum disorder (FASD), which refers to the broad range of deficits associated with alcohol exposure

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Research in context

Evidence before this study

Prenatal alcohol exposure has been shown to impact the development and function of a wide range of physiological systems, including the immune system. And despite results from a recent health survey spearheaded by young adults with fetal alcohol spectrum disorder (FASD) indicating that autoimmune conditions may occur at higher rates in individuals with FASD, there has been minimal investigation of the impact of prenatal alcohol exposure on the incidence and pathophysiology of autoimmune disorders.

Added value of this study

Our results indicate that, in a rodent model of rheumatoid arthritis, alcohol-exposed animals display an increased incidence and severity of joint inflammation, as well as an impaired recovery from inflammation, as compared to unexposed control animals. In addition, the combination of alcohol-exposure and adolescent stress exposure resulted in increased signs of joint inflammation

Implications of all the available evidence

These data support the recent health survey data indicating that rates autoimmune conditions, such as rheumatoid arthritis, may be higher following prenatal alcohol exposure, and further highlight that the pathophysiology of these conditions may be different following prenatal alcohol exposure. As the North American prevalence of FASD is estimated at approximately 1.1–5.0%, there is a large number of individuals with FASD who may be experiencing or at higher risk of developing autoimmune conditions. In addition, it is unclear given the potential differences in pathophysiology, whether currently available treatments for autoimmune conditions, such as rheumatoid arthritis, will be effective or well tolerated in this population. As such, additional clinical research examining the pathophysiology of autoimmune conditions in individuals with FASD is warranted.

during pregnancy in humans. FASD prevalence is estimated at 1% in some populations⁵ and up to as high as 5% in others.⁶ While there is extensive evidence for immune disturbances following PAE, such as an increased incidence of major and minor infections, alterations in immune organ development, and decreased immune cell responses to challenge,⁷ and more recently, data from our group demonstrating evidence of increased susceptibility to experimentally-induced arthritis,⁸ much less is known regarding immune disturbances and the incidence of autoimmune disorders in FASD. Recently, however, results from an informal health survey initiated by young adults with FASD suggest that PAE may result in a

heightened vulnerability to autoimmune disorders, including rheumatoid arthritis (estimated prevalence at 7.2%).⁹ This highlights the importance of further investment into understanding the pathophysiology of autoimmune disorders, such as rheumatoid arthritis, in PAE animal models and the translation of these findings to humans to better understand how alcohol exposure may be impacting the incidence and severity of autoimmune disorders in this population. Briefly, as a model of human rheumatoid arthritis, here we used the rodent adjuvant-induced arthritis model which is a rapid onset, T-cell dependent inflammatory model that results in inflammation of the joints (in approximately 40–60% of animals), and in particular the hind paw joints, beginning approximately 10 days post-injection and peaking around 16 days post-injection (with variation based on rat strain, dose, and mode of injection).^{10–13} Histopathological and radiological examination of hind paw joints generally identifies joint damage and deformity by day 15–30 post-injection,¹⁴ with the active chronic inflammation phase generally lasting approximately 35 days.¹³

It has been well established that for autoimmune disorders such as rheumatoid arthritis there is altered communication between the hypothalamic-pituitary-adrenal (HPA) axis and the immune system and that this may contribute to disease onset and trajectory. Specifically, while the HPA response to stressors may be relatively normal, inability to mount an appropriate HPA response to inflammation, resulting in impaired ability to inhibit ongoing inflammation is suspected in at least a subset of patients with rheumatoid arthritis.^{15,16} Importantly, alterations in HPA regulation and responsiveness have been well described in individuals with FASD¹⁷ and in PAE animal models.¹⁸ As well, children with FASD are at a heightened risk of experiencing early-life stress due to foster care placements, childhood abuse and neglect, and other adverse early life circumstances,^{19,20} which are known to impact both HPA²¹ and immune²² function. Investigations into altered HPA-immune interactions are thus particularly relevant for elucidating mechanisms underlying the increase in autoimmune disorders following PAE.

Here, we utilized a “multiple-hit” or cumulative stress concept to assess possible independent and interactive effects of PAE and adolescent stress on arthritis outcomes. This concept suggests that adverse experiences during early life, in this case, alcohol exposure (first hit), program physiological systems, resulting in altered HPA/immune sensitivity and increased responsiveness to later-life challenges (second and third hits). Thus, following PAE, half the rats were exposed to chronic mild stress (CMS) and half remained undisturbed during adolescence. Building on our previous finding that PAE results in a more severe and prolonged arthritis course,⁸ we terminated animals at the peak of inflammation and following recovery. We hypothesized that PAE would

result in more severe arthritis and impaired recovery from arthritis, which would be further exacerbated by adolescent stress. Furthermore, we expected a mismatch among arthritis severity, HPA activity, and joint integrity and inflammation in PAE rats. Utilization of clinically relevant measures (hormone and immune markers and histopathology of the tibiotarsal joint) enabled us to gain insight into factors predictive of the heightened sensitivity to inflammation in PAE rats. Importantly, insight into the physiological underpinnings of arthritis in the PAE model may lead not only to improved treatment for individuals with FASD, but also to a broader understanding of environmental risk factors of rheumatoid arthritis.

Methods

Breeding and prenatal diets

Adult female Sprague-Dawley rats (50–60 days old) were obtained from Charles River Laboratories (St. Constant, Québec, Canada; $n = 40$; RRID:RDG_734476). Following a two-week acclimatization period, female Sprague-Dawley rats (minimum weight: 250 g) were pair-housed with males (minimum weight: 330 g) and vaginal lavage samples collected daily to check for sperm, indicating gestation day 1 (GD1). On GD1, dams were single housed and assigned to: Prenatal alcohol exposure (PAE; $n = 17$)—*ad libitum* access to liquid ethanol diet (36% ethanol-derived calories, 6.37% v/v; or Control (C; $n = 23$)—pelleted version of the liquid control diet, *ad libitum* [Weinberg/Keiver High Protein Ethanol (#710324) and Pelleted Control (#102698) Diet; Dyets Inc. Bethlehem, PA]. Blood alcohol levels were measured as previously reported^{23,24} and ranged from ~90 to 150 mg/dl. On GD21, experimental diets were replaced with laboratory chow. On postnatal day 1 (P1), litters were culled to 6 males and 6 females. One female per litter, per testing condition, was used in the study to control for litter effects.

Adolescent exposure to chronic mild stress (CMS)

On P31, rats were weighed and a basal blood sample was collected from the tail vein (0800–1000 h). Rats were then assigned to one of two stress conditions: (1) the non-CMS (no stress) or (2) the CMS (stress) group. CMS consisted of ten days of randomized stress exposure. Stressors included: elevated platform, cage tilt, novel cage, soiled cage, restrain, social isolation, water deprivation as per.²⁵ On P41, all rats were weighed and a basal blood sample collected. Body weight and corticosterone levels before and after the CMS period are presented in Supplementary Table 1.

Clinical evaluation of arthritis and tissue collection

On P55–60, rats received a single intradermal injection of either 0.6 mg complete Freund's adjuvant [CFA/

adjuvant; *Mycobacterium tuberculosis* H37 RA dissolved in incomplete Freund's adjuvant (Difco laboratory, Detroit, MI); $n = 28$ /prenatal treatment/stress condition] or saline ($n = 12$ /prenatal treatment/stress condition) at the base of the tail under isoflurane anaesthesia as previously reported.^{26–28} Briefly, anaesthesia involved placing rats in an induction chamber and exposing them to isoflurane and oxygen and transferring them to a nose cone in order to either receive an injection or assess the paws (average amount of time under anaesthesia: <5 min per exposure). To assess arthritis severity, rats were again briefly anesthetized, weighed, and clinical signs of arthritis (clinical score) assessed on days 5, 9, 11, 13, 15 post-injection, and every three days thereafter, up to 57 days post-injection (saline-injected rats were anesthetized in parallel with their adjuvant-injected counterparts). To calculate the clinical score, each of the four paws was scored on a 0–4 point scale, where 0 = no signs of arthritis, 1 = single focus of redness or swelling, 2 = two or more foci of redness or swelling, 3 = confluent but not global swelling, 4 = severe global swelling. Total clinical scores were obtained by summing the clinical scores for each paw as previously reported.^{26–28}

Following adjuvant-injection, animals were monitored daily in the home cage for signs of pain, discomfort or infection, as well as for general signs of health including alertness, activity, coat quality, colour of the ears, and ability to rear. At the onset of arthritis, pain levels were assessed twice daily in animals with clinical scores ≥ 8 . Pain was scored from 0 (pain free; animal is active alert, well-groomed and healthy) to 4 (severe pain: animal displays signs including swelling of the face, presence of porphyrin, hunched back stance, decreased coat condition, sunken eyes and animal is unresponsive to cage disturbance and is reluctant to move). Rats achieving an elevated score (≥ 3) were administered buprenorphine (0.03 mg/kg) and subcutaneous fluids twice daily. Rats that maintained an elevated pain score for 2 days (humane endpoint) were terminated under basal conditions, when possible.

Two overlapping cohorts were run: (1) terminated on day 16 post-injection [*induction-to-peak phase*; saline: $n = 6$ /prenatal treatment/stress condition; adjuvant-injected: $n = 14$ /prenatal treatment/stress condition]; (2) terminated following recovery from arthritis [clinical score = 0 for ten days post-injection – *resolution phase*; saline: $n = 6$ /prenatal treatment/stress condition; adjuvant-injected: $n = 14$ /prenatal treatment/stress condition]. Adjuvant-injected rats were categorized as either: Adj/AA – Adjuvant-injected/Active arthritis (clinical signs of arthritis during the experimental period; clinical score ≥ 1), Adj/NA – Adjuvant-injected/No clinical signs of arthritis throughout the experimental period (clinical score = 0), or Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain). On each termination day rats were quickly decapitated

(0800–1030 h) and trunk blood and hind paws collected as per,²⁶ with paws stored in 10% buffered formalin. Vaginal lavage samples were also collected for oestrus cycle staging. Researchers were blinded to prenatal treatment group, stress condition, and arthritis group, whenever possible (i.e. following prenatal diet administration period, conclusion of the stress paradigm).

An *a priori* power analysis was computed using G*Power (3.1.9.3, Heinrich-Heine-University Düsseldorf). To achieve a large effect size in the model (effect size f : 0.35) at a power of 0.80 and α = 0.05, the total sample size was estimated at 67 animals per time point (peak of arthritis, resolution phase; actual sample size: 80 animals/time point)

Plasma hormone, CBG and immune measurements

Total corticosterone and oestradiol levels were measured by radioimmunoassay (RIA), using standard protocols [ImmuChem Double Antibody Corticosterone ¹²⁵I RIA kit (MP Biomedicals, LLC, Orangeburg, NY, USA); Ultra-Sensitive Estradiol RIA (Beckman Coulter, Mississauga, Ontario, Canada)]. The minimum detectable concentrations were 7.7 ng/mL and 2.2 pg/ml for corticosterone and estradiol, respectively. Intra- and inter-assay coefficients of variation were <10% for all RIA's. The steroid-binding capacity of corticosteroid binding globulin (CBG, an additional measure of HPA regulation) was measured using a ligand-saturation assay that uses dextran-coated charcoal to separate CBG-bound from free [³H]-corticosterone (PerkinElmer Lifer Sciences, Waltham, MA) as per^{29,30} (dilution: 1:1500). C-reactive protein (CRP) was measured as previously reported³¹ with a lower limit of detection of 2.22–3.23 pg/ml.

Tibiotarsal joint sectioning, staining, and histopathological evaluation

Hind paws were decalcified and dehydrated in graded ethanols and embedded in paraffin wax. Midaxial paw sections (5 μ m) were collected using a microtome (Leica Microsystems, Concord, Ontario, Canada). Slides were stained with haematoxylin and eosin (H&E; n = 3/rat) or toluidine blue (n = 1/rat). Dehydration, embedding, slicing, and H&E staining were performed by Wax-It Histology Services Inc. (Vancouver, BC, Canada). H&E stained sections were scored for soft tissue inflammation, bone erosion, bone marrow activity, skeletal muscle integrity, and synovial tissue status based on published histopathological assessment criteria^{32–34} (scoring criteria available in Supplementary Table 2). Cartilage thickness was assessed at the level of the distal tibia by measuring the distance between the joint space and the edge of the cartilage at the inferior edge of the tibia using ImageJ (National Institutes of Health, Bethesda, MD). Representative images are shown in Figure 5 (H&E) and Figure 6 (cartilage).

CD163 immunohistochemistry (IHC) for synovial macrophage density and CD163⁺ chondrocyte analysis

Slides (n = 1/rat) were stained for CD163, according to the following procedure: Slides were placed into an incubator (50°C) for 30 min, and next taken through a series of xylenes and ethanols to deparaffinize the tissue. Slides were then washed in TBS and sections outlined using a hydrophobic pen. Slides were again washed in TBS and taken through antigen retrieval through incubation in 10 μ g/ml proteinase K in TBS for 10 min in a 37 °C water bath. Next, sections were washed in TBS, followed by the addition of 400 μ l of blocking buffer (TBS-T + 4% Normal Horse Serum) to each slide, for 3 h. Primary antibody (commercially available, validated mouse anti-rat CD163; clone ED2, Serotec, cat# MCA342GA, Raleigh, NC; 1:100) was prepared in blocking buffer with 400 μ l applied to each slide and incubated overnight at 4°C on a shaker. Following a 17,18 h incubation in primary antibody, slides were rinsed in TBS-T (x3) and secondary antibody applied (biotinylated horse anti-mouse IgG antibody, rat adsorbed (Vectorlabs, Burlingame, CA) in blocking buffer for 1 h at room temperature. Next, slides were rinsed in TBS and alkaline phosphatase streptavidin (APS; 1:500) in blocking buffer applied to the slides for 30 min. Slides were next washed in TBS and a NBT/BCIP working solution added to slides, protected from light, for 30 min. Finally, slides were washed in TBS, distilled water, and dehydrated in graded ethanols, followed by xylene, and coverslipped with permount. Using ImageJ, a threshold was applied to the CD163 slides, and Image Overlay Utility (G. Keller) was used to superimpose the H&E and CD163 sections, allowing for the synovium to be traced and the signal measured using ImageJ. Representative images are shown in Figure 7.

Tibiotarsal joint imaging

H&E sections were assessed using an Axioskope mot plus microscope (Zeiss, Toronto ON). Scans of whole hind paw sections (maximum resolution 20x) were acquired using an Aperio ScanScope CS slide scanner (Leica Biosystems, Concord, ON). High resolution images (Figures 5e–h; 6e–h) were generated by zooming in on the whole paw scans using Aperio ImageScope v12.2.2.5015 (Leica Biosystems, Buffalo Grove, IL). Images of cartilage and CD163⁺ staining (Figure 7e–m) were captured using a QIClick CCD Camera (QImaging, Surrey, BC) and processed using Northern Eclipse version 8 (Empix, Mississauga, ON).

Statistical analyses

Data were analysed using analysis of variance (ANOVA) for the factors of prenatal treatment, stress condition, and arthritis severity, as appropriate, followed by Fisher

post hoc tests (significant ANOVA *p* values and *F* statistics reported in text, *post hoc p* values reported in figures) (IBM SPSS Statistics). Oestrus stage was included as a covariate in the oestradiol analyses but was not significant. Arthritis incidence, recovery from arthritis, and achievement of humane endpoint were coded as yes/no variables and were analysed by logistic regression for the factors of prenatal treatment and stress condition. Differences were considered significant at $p \leq 0.05$. Corticosterone, oestradiol, CBG, and CRP levels were transformed using the Blom rank-based normalization method for statistical analyses with untransformed data presented in the figures for clarity.

During the resolution phase, a small number of rats reached humane endpoint or failed to resolve from arthritis (non-CMS: C, $n = 1$, PAE, $n = 2$; CMS: C, $n = 3$, PAE, $n = 5$). While these cases are included in the analysis of arthritis incidence, severity, and recovery, endocrine and immune parameters and histological assessment data for these cases were excluded. Overall, in this subgroup, endocrine and immune measures paralleled the responses observed on day 16 with active arthritis (Adj/AA), as all of these rats continued to show clinical signs of arthritis. Other than these animals with ongoing arthritis in the recovery group, there were no other exclusions.

A table summarizing the statistically significant main effects and interactions is available in Supplementary Table 3.

Ethics

All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Canadian Council on Animal Care (CCAC) and approved by the University of British Columbia Animal Care Committee (certificate: Prenatal Alcohol and Neuroimmunity A12-0032 3/19/2012 – 3/20/2016).

Role of funding sources

The funders had no role in study design, data collection, data analysis, interpretation, writing of the manuscript, or in the decision to submit the paper for publication.

Results

Arthritis incidence and severity

Arthritis incidence and severity was assessed throughout the course of the experiment on days 6, 9, 11, 13, and 15 post-injection for the induction-to-peak phase and every three days thereafter (up to 57 days post-injection) for the resolution phase. This assessment was made through visual inspection of the four paws while the animal was under anaesthesia, allowing for the date of arthritis onset to be identified, as well as a time course of arthritis progression to be described (Figure 1).

Arthritis incidence (% of rats with clinical signs of arthritis) is depicted in Figure 2a,b. In the regression model, arthritis incidence did not differ among groups in the induction-to-peak phase. In the resolution phase, however, an association between arthritis incidence and prenatal treatment was detected. The odds of developing arthritis with adjuvant injection increased by 1.95 for PAE compared to control rats ($p = 0.016$; 95% confidence interval for odds ratio: 1.13, 3.38). Logistic regression to examine the probability of recovery from arthritis or achievement of humane endpoint during the resolution phase found no significant associations.

Average clinical scores from post-injection day 11–15 and day 11–57 are depicted in Figure 2c and d, respectively. As expected, there was an overall increase in clinical scores from days 11 to 15 post-adjuvant injection [ANOVA, $F_{(1, 104)}=25.93$, $p < 0.001$], with no differences among groups (Figure 2c). During the resolution phase, however, PAE and C rats showed a differential pattern of change over days, regardless of stress condition [ANOVA, interaction: prenatal treatment \times day, $F_{(16, 752)}=2.71$, $p < 0.05$]; PAE rats had higher clinical scores than C rats from days 18 to 57 post-injection, with the exception of days 42 and 45 (Figure 2d).

Analysis of the clinical scores through area under the curve showed, again, no differences between groups in the induction-to-peak phase (Figure 2e) but during the resolution phase, area under the curve was greater in PAE compared to C rats, regardless of stress condition (Figure 2f) [ANOVA, main effect of prenatal treatment: $F_{(1, 56)}=7.42$, $p < 0.01$].

Hormone and immune measures

Corticosterone and CBG. An altered HPA response to inflammation is suspected in at least a subset of patients with rheumatoid arthritis.^{15,16} HPA axis dysregulation also commonly occurs with PAE³⁵ and as such, here we explored whether altered HPA responses to inflammation would underlie, at least in part, the differential course of arthritis between PAE and C animals. At the peak of arthritis, basal corticosterone was higher in adjuvant-injected (Adj/NA, Adj/AA) compared to saline-injected rats [ANOVA, main effect of arthritis severity: $F_{(2, 68)}=5.95$, $p < 0.01$], with no differential effects by prenatal treatment or stress condition (Figure 3a). At resolution, by contrast, prenatal treatment was found to modulate the effect of arthritis severity on corticosterone [ANOVA, prenatal treatment \times arthritis severity interaction: $F_{(2, 56)}=3.74$, $p < 0.05$], with higher corticosterone levels detected in PAE rats that had recovered from arthritis (Adj/Rec), compared to their control counterparts (Figure 3b).

Next, examination of CBG at the peak of arthritis revealed a differential pattern by stress condition in an

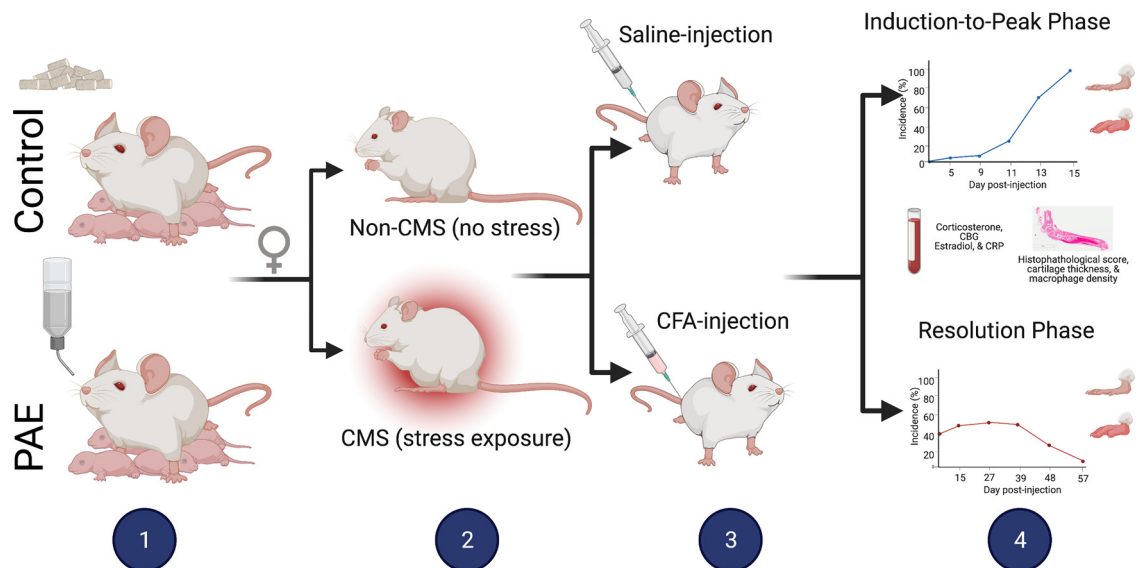


Figure 1. Study overview

1: Diets (liquid ethanol or pelleted control) were administered to pregnant dams throughout gestation (GD 1 - 21); 2: Adolescent females were exposed to chronic mild stress (CMS) or left undisturbed (non-CMS) from P31 - 41; 3: Adult rats (P55 - 60) received a single injection of complete Freund's adjuvant (CFA) or saline intradermally at the base of the tail; 4: Arthritis severity was assessed on days 5, 9, 11, 13, 15 post-injection and every three days thereafter. Rats were terminated either at the peak of arthritis (day 16 post-injection) or followed through resolution (terminated following recovery from arthritis). Levels of corticosterone, CBG, oestradiol, and CRP were measured in plasma and arthritis severity (histopathological score), cartilage thickness, and macrophage density assessed in the hind paws of all animals.

PAE: prenatal alcohol exposure; CMS: chronic mild stress; CRP: C-reactive protein. Created with BioRender.com

arthritis severity-dependent manner [ANOVA, stress condition \times arthritis severity interaction: $F_{(2, 67)}=3.80$, $p < 0.05$]. As expected, CBG levels decreased in adjuvant-injected (Adj/NA, Adj/AA), compared to saline-injected rats in the non-CMS condition, but interestingly, only decreased in rats with active arthritis in the CMS condition (Figure 3c). In addition, CBG levels were lower overall in PAE rats compared to control rats in the non-CMS condition [ANOVA, prenatal treatment \times stress condition interaction: $F_{(1, 67)}=5.73$, $p < 0.05$]. At resolution, however, CBG levels were modulated by prenatal treatment, stress condition, and arthritis severity [ANOVA, prenatal treatment \times stress condition \times arthritis severity interaction: $F_{(2, 59)}=5.00$, $p < 0.01$]. In the non-CMS condition, PAE rats that recovered from arthritis (Adj/Rec) had lower CBG levels compared to saline-injected PAE rats, and compared to their C counterparts (Adj/Rec). In the CMS condition, by contrast, there was a drop in CBG in all adjuvant injected (Adj/NA, Adj/Rec) control but not PAE rats (Figure 3d).

Oestradiol. Oestrogens have consistently been shown to play a protective role in rheumatoid arthritis³⁶ and in animal models, manipulation of the hormonal milieu through ovariectomy or oestradiol injection has a

dramatic effects on disease course.³⁷ As such, here we explored whether there was a relationship between oestradiol levels and arthritis disease status. At the peak of arthritis, oestradiol levels decreased with adjuvant injection (Adj/NA, Adj/AA), across prenatal treatments and stress conditions [ANOVA, main effect of AA severity: $F_{(2, 63)}=9.24$, $p < 0.001$] (Figure 3e). At resolution, however, oestradiol was highest in rats that failed to develop clinical signs of arthritis (Adj/NA) [ANOVA, main effect of arthritis severity: $F_{(2, 58)}=4.19$, $p < 0.05$] (Figure 3f). In addition, rats in the CMS condition had higher oestradiol levels overall, compared to the non-CMS condition [ANOVA, main effect of stress condition: $F_{(1, 106)}=6.86$, $p = 0.010$] (Figure 3f).

CRP. CRP is an acute-phase protein that has been well-established to increase in response to inflammation. In the context of rheumatoid arthritis, CRP levels begin to increase prior to the onset of symptoms³⁸ and long-term elevations in CRP are associated with greater joint damage.³⁹ At the peak of arthritis, prenatal groups showed differential levels of CRP by arthritis condition [ANOVA, prenatal treatment \times arthritis severity interaction: $F_{(2, 66)}=4.13$, $p < 0.020$]. In control rats, CRP increased with clinical signs of arthritis (Adj/AA), whereas in PAE rats, CRP levels also increased in

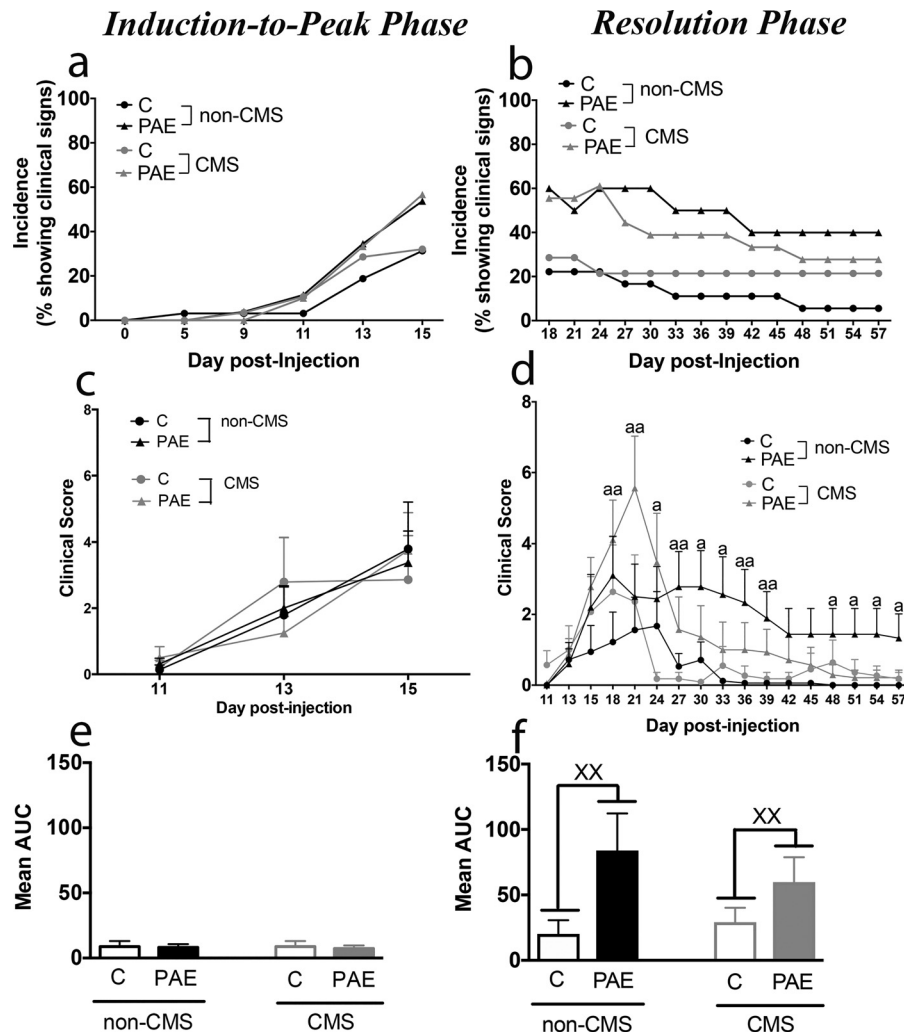


Figure 2. Clinical course of arthritis

a,b: Incidence of arthritis as the percentage of adjuvant-injected rats showing clinical signs of arthritis during the induction-to-peak phase (a) and during the resolution phase (b). c,d: Mean clinical scores (\pm SEM) for rats that developed arthritis during the induction-to-peak phase (c) and during the resolution phase (d). e,f: Area under the curve (AUC; mean \pm SEM) of the average clinical scores during the induction-to-peak phase (e) and the resolution phase (f). During the resolution phase, AUC was higher in PAE compared to C rats across stress conditions. "a": prenatal treatment \times day interaction; "x": main effect of prenatal treatment. Statistical test (a–d): ANOVA; e,f: area under the curve (AUC). Significant *post hoc* comparisons: One symbol: $p < 0.05$; Two symbols: $p < 0.01$; Saline: $n = 6$ /prenatal treatment/stress condition/time point; Adjuvant-injected: $n = 14$ /prenatal treatment/stress condition/time point. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition.

adjuvant-injected rats that failed to develop clinical signs of arthritis (Adj/NA). Interestingly, with arthritis onset (Adj/AA), however, CRP levels were lower in PAE compared to control rats (Figure 4a). At resolution, CRP was not different among groups (Figure 4b).

Histopathological evaluation of tibiotarsal joints by H&E staining

In addition to the description of arthritis incidence and severity through visual inspection of the paws as

described above, at termination at either the peak of arthritis or following recovery from arthritis, hind paws were collected such that the integrity of the tibiotarsal joint and surrounding tissue could be evaluated. Importantly, this histopathological evaluation allowed for a more comprehensive analysis of arthritis severity, including differentiation between oedema and bone and cartilage damage.

At the peak of arthritis, as expected, total histopathological score was highest in rats exhibiting active arthritis (Adj/AA), across prenatal treatments and stress

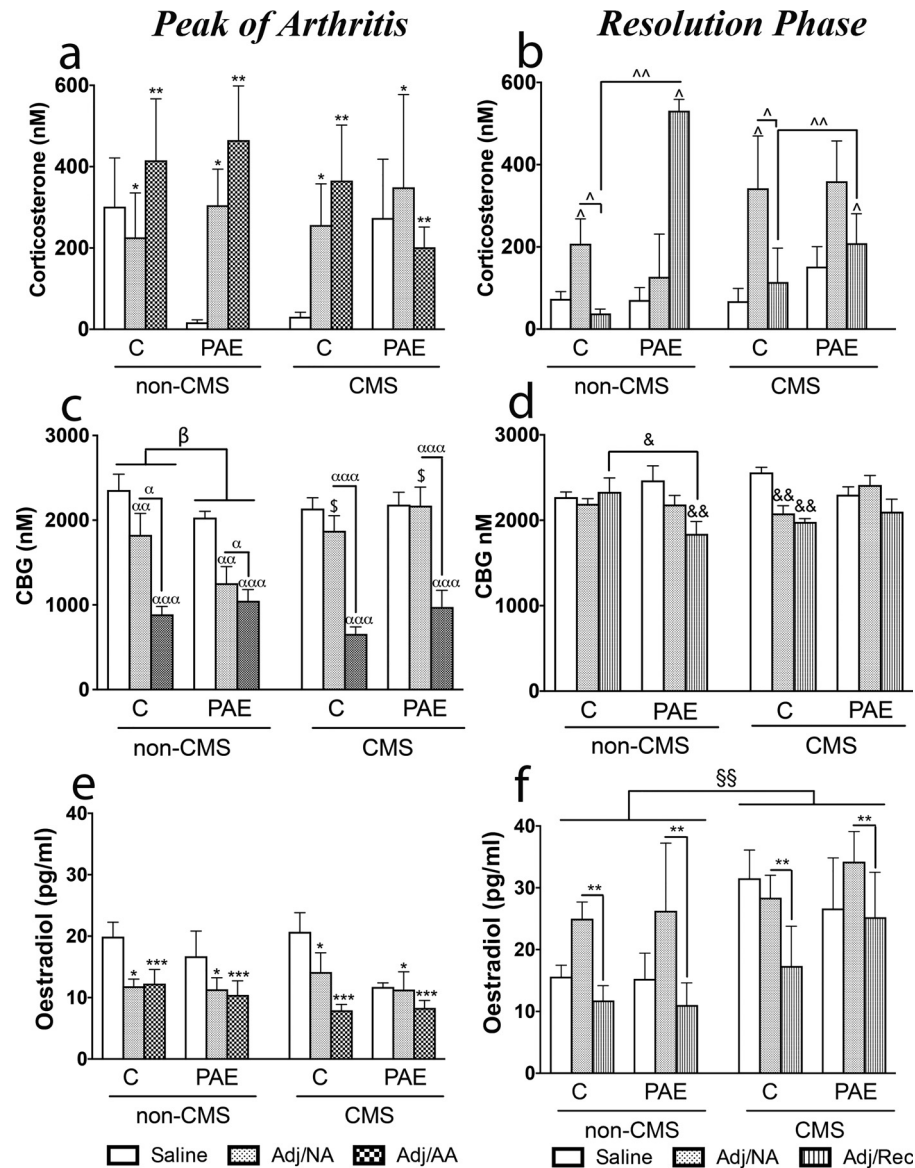


Figure 3. Corticosterone, corticosteroid binding globulin (CBG), and oestradiol levels

Bars represent mean hormone level \pm SEM. Data are presented in raw form, with statistical analyses conducted on Blom transformed (normalized) data.

a: At the peak of arthritis, corticosterone was higher in adjuvant-injected (Adj/NA, Adj/AA) compared to saline-injected rats, across prenatal treatment and stress conditions. “***”: main effect of arthritis severity. b: During the resolution phase, corticosterone was higher in PAE rats that recovered from arthritis (Adj/Rec), compared to their saline-injected counterparts. In addition, PAE rats that recovered from arthritis (Adj/Rec) had higher corticosterone than their control counterparts (Adj/Rec). In C rats, corticosterone was higher in rats that failed to develop clinical signs of arthritis (Adj/NA), compared to saline-injected rats and rats that developed arthritis (Adj/AA). “Λ” prenatal treatment \times arthritis severity interaction. c: At the peak of arthritis, in the non-CMS condition, CBG was lower in adjuvant-injected (Adj/NA, Adj/AA), compared to saline-injected rats. In addition, CBG was lower in rats that developed arthritis (Adj/AA), compared to rats that failed to develop clinical signs of arthritis (Adj/NA). CBG was also lower overall in PAE compared to C non-CMS rats. In the CMS condition, CBG was again lower in rats that developed arthritis (Adj/AA), compared to saline-injected rats. In addition, rats that developed arthritis (Adj/AA) also had lower CBG than rats that failed to develop clinical signs of arthritis (Adj/NA). Finally, CBG was higher in CMS rats that failed to develop clinical signs of arthritis (Adj/NA) compared to their non-CMS counterparts (“\$”). “α”: stress condition \times arthritis severity interaction. “β”: prenatal treatment \times stress condition interaction. d: During the resolution phase, in the non-CMS condition, CBG was lower in PAE rats that recovered from arthritis (Adj/Rec), compared to their saline-injected counterparts and compared to C rats that recovered from arthritis (Adj/Rec). In the CMS condition, in C rats only, CBG was decreased with adjuvant-injection (Adj/NA, Adj/AA). “&”: prenatal treatment \times stress condition \times arthritis

conditions [ANOVA, main effect of arthritis severity: $F_{(2, 67)}=34.30$, $p < 0.001$] (Figure 5a). At resolution, however, total histopathological score differed by prenatal treatment, stress condition, and arthritis severity [ANOVA, prenatal treatment \times stress condition \times arthritis severity interaction: $F_{(2, 55)}=3.14$, $p < 0.05$]. Specifically, in the non-CMS condition, while there were no differences in the total histopathological score by arthritis severity condition for control rats, PAE rats that recovered from arthritis (Adj/Rec) had the highest total histopathological score (Figure 5b). By contrast, in the CMS condition, for both C and PAE rats, total histopathological score was increased in rats that recovered from arthritis (Adj/Rec), compared to saline-injected rats. Finally, despite no external signs of inflammation, adjuvant-injected PAE rats (Adj/NA) had higher histopathological scores compared to their saline-injected counterparts (Figure 5b). Whole paw scans showing representative H&E staining in a saline-injected and adjuvant-injected rat are shown in Figure 5c,d. Figure 5e–h show the tibiotarsal joint in a saline-injected and an adjuvant-injected rat.

Cartilage thickness

The integrity of the articular cartilage is commonly used to assess arthritis severity, with cartilage damage indicative of a longer and more severe arthritis progression. As such, here we measured cartilage thickness at the level of the distal tibia in order to quantify the degree cartilage involvement.

At the peak of arthritis, as expected, cartilage thickness decreased in rats that developed arthritis (Adj/AA) compared to saline-injected and adjuvant-injected rats that failed to develop arthritis (Adj/NA), across prenatal treatments and stress conditions [ANOVA, main effect of arthritis severity: $F_{(2, 66)}=6.06$, $p < 0.01$] (Figure 6a). At resolution, however, PAE rats had increased cartilage thickness overall compared to controls, regardless of stress condition and arthritis severity [main effect of prenatal treatment: $F_{(1, 57)}=8.08$, $p < 0.01$] (Figure 6b). Whole paw scans showing representative cartilage staining in a saline-injected and adjuvant-injected rat are shown in Figure 6c,d. Figure 6e, g and f, h show the tibiotarsal joint in a saline-injected and an adjuvant-injected rat, respectively.

Macrophage density in the synovium and CD163⁺ chondrocyte staining in the cartilage

Macrophages may be particularly vulnerable to early-life insults due to their distribution at “portals of environmental exposure”,⁴⁰ including the airway, and gastrointestinal tract. With rheumatoid arthritis, macrophages levels increase in the synovium and pannus and due to their proinflammatory properties, greatly contribute to inflammation and joint damage throughout the disease course.⁴¹ As such, here we quantified macrophage levels based on the hypothesis that elevated histopathological scores could be linked to increased macrophage infiltration into the joint.

At the peak of arthritis, as expected, macrophage (CD163⁺) density was highest in rats that developed arthritis (Adj/AA), across prenatal treatments and stress conditions [ANOVA, main effect of arthritis severity: $F_{(2, 63)}=5.32$, $p < 0.01$] (Figure 7a). At resolution, however, macrophage density was impacted by prenatal treatment and stress condition, in an arthritis severity-dependent manner [ANOVA, prenatal treatment \times stress condition \times arthritis severity interaction: $F_{(2, 58)}=5.28$, $p < 0.01$]. Specifically, in the CMS condition, PAE rats that recovered from arthritis (Adj/Rec) showed the highest overall macrophage density. Whole paw scans showing representative macrophage staining in a saline-injected and adjuvant-injected rat are shown in Figure 7c–d. Figure 7e–m shows CD163⁺ chondrocyte staining (e, h, k), cartilage staining (f, i, l), and the overlay between the two, with areas of cartilage thickening characterized by increased CD163⁺ chondrocyte staining.

Discussion

The current data support and significantly extend our previous findings demonstrating a modulatory role of PAE on the inflammatory course and disease profile of adjuvant-induced arthritis. PAE rats had an increased incidence and severity of arthritis, in support of our previous findings,⁸ as well as significantly impaired recovery from arthritis. Evaluation of the hormonal milieu revealed that PAE rats display changes in the corticosterone/CBG balance supporting a role for CBG in their differential arthritis course. In addition, increased joint damage, as measured by the histopathological score,

severity interaction. e: At the peak of arthritis, oestradiol was lower in all adjuvant-injected (Adj/NA, Adj/AA) compared to saline-injected rats, across prenatal treatment and stress conditions. “*”: main effect of arthritis severity. f: During the resolution phase, oestradiol was higher in rats that failed to develop clinical signs of arthritis (Adj/NA), compared to rats that recovered from arthritis (Adj/Rec), across prenatal treatment and stress conditions. In addition, oestradiol levels were higher overall in CMS, compared to non-CMS rats. “*”: main effect of arthritis severity. “§”: main effect of stress condition. Statistical test (a–f): ANOVA; Significant *post hoc* comparisons: One symbol: $p < 0.05$; Two symbols: $p < 0.01$; Three symbols: $p < 0.001$; Saline: $n = 6$ /prenatal treatment/stress condition/time point; Adjuvant-injected: $n = 14$ /prenatal treatment/stress condition/time point. All assays were performed in duplicate. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition; Saline: saline-injected; Adj/AA: Adjuvant-injected/Active arthritis (clinical score ≥ 1); Adj/NA: Adjuvant-injected/No clinical signs of arthritis; Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain)

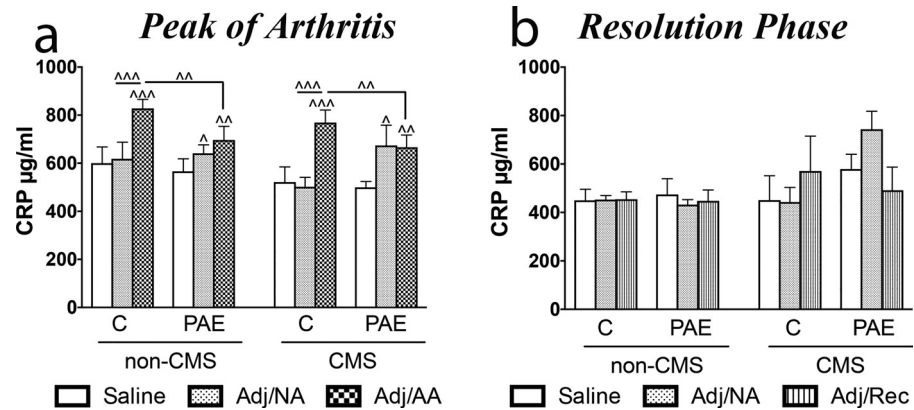


Figure 4. Plasma C-reactive protein (CRP) levels

Bars represent mean CRP level \pm SEM. Data are presented as raw CRP levels (pg/ml) with statistical analyses conducted on Blom transformed (normalized) data.

a: At the peak of arthritis, CRP was elevated in rats that developed arthritis (Adj/AA), compared to saline-injected rats, across prenatal treatment and stress conditions. In C rats only, CRP was also higher in rats that developed arthritis (Adj/AA), compared to rats that failed to develop clinical signs of arthritis (Adj/NA). In PAE rats only, rats that failed to develop clinical signs of arthritis (Adj/NA) also had higher CRP levels than their saline-injected counterparts. Finally, CRP levels were lower in PAE compared to C rats that developed arthritis (Adj/AA). “^”: prenatal treatment \times arthritis severity interaction. Statistical test (a,b): ANOVA; Significant *post hoc* comparisons: ^: $p < 0.05$; ^^: $p < 0.01$; ^^: $p < 0.001$; Saline: $n = 6$ /prenatal treatment/stress condition/time point; Adjuvant-injected: $n = 14$ /prenatal treatment/stress condition/time point. CRP assays were performed in duplicate. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition; Saline: saline-injected; Adj/AA: Adjuvant-injected/Active arthritis (clinical score ≥ 1); Adj/NA: Adjuvant-injected/No clinical signs of arthritis; Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain)

following resolution of clinical signs of arthritis, provides further evidence of greater inflammation in PAE compared to control rats, while it appears that oestradiol may have a protective role. Moreover, the addition of adolescent stress resulted in increased macrophage density in the synovium of PAE rats that appeared to have resolved from arthritis (Adj/Rec), a finding that was unique to the PAE group. Taken together, findings in PAE rats demonstrating unique and interactive effects of alcohol-exposure and adolescent stress on key endocrine, immune, and histopathological parameters are suggestive of a significant immunomodulatory role of early environmental exposures/conditions. As there is a scarcity of clinical data in children and adults with FASD, further studies are needed to explore this issue in human populations.

Our data on recovery from arthritis indicate that 40% of PAE rats failed to recover from arthritis, which is approximately double the total incidence of arthritis in control rats. Interestingly, in PAE rats, impaired resolution was observed for those in the non-stress (non-CMS) condition, while exposure to adolescent stress (CMS) appeared to accelerate recovery. Of note, however, while external signs of inflammation (i.e., clinical scores) resolved at the level of the tibiotarsal joint, histopathological scores were elevated and macrophage density was increased in animals exposed to PAE and adolescent stress. Taken together, this is suggestive of ongoing internal inflammation or failure to recover

from inflammation at the tissue and cellular level, despite a lack of external signs of arthritis.

Inappropriate activation of the HPA axis in the context of ongoing inflammation is thought to be implicated in the pathophysiology of at least a subset of rheumatoid arthritis cases.¹⁵ In support of this possibility, we have shown previously, in control rats, that lower basal levels of CBG, the major transport protein for glucocorticoids, likely plays a critical role in the risk of arthritis onset, as lower CBG signals a reduction in the corticosterone reservoir available to target sites of inflammation.²⁶ Our present data support a role for CBG in the differential arthritis course in PAE compared to control rats – CBG levels decreased with arthritis onset in rats in the non-stress condition; however, there was an unexpected drop in CBG levels with adjuvant injection in rats that did not develop clinical signs of arthritis (Adj/NA), which appears to be driven primarily by a more marked drop in CBG for PAE rats. We propose that a drop in CBG prior to the onset of arthritis may represent a maladaptive response such that PAE rats cannot further cleave CBG to release the corticosterone needed to dampen inflammation. In addition, following resolution from arthritis, we detected an altered corticosterone/CBG profile in non-stress PAE rats – increased corticosterone in conjunction with decreased CBG. This HPA profile suggests that despite the appearance of resolution, as measured by external signs of inflammation, PAE rats are likely experiencing ongoing

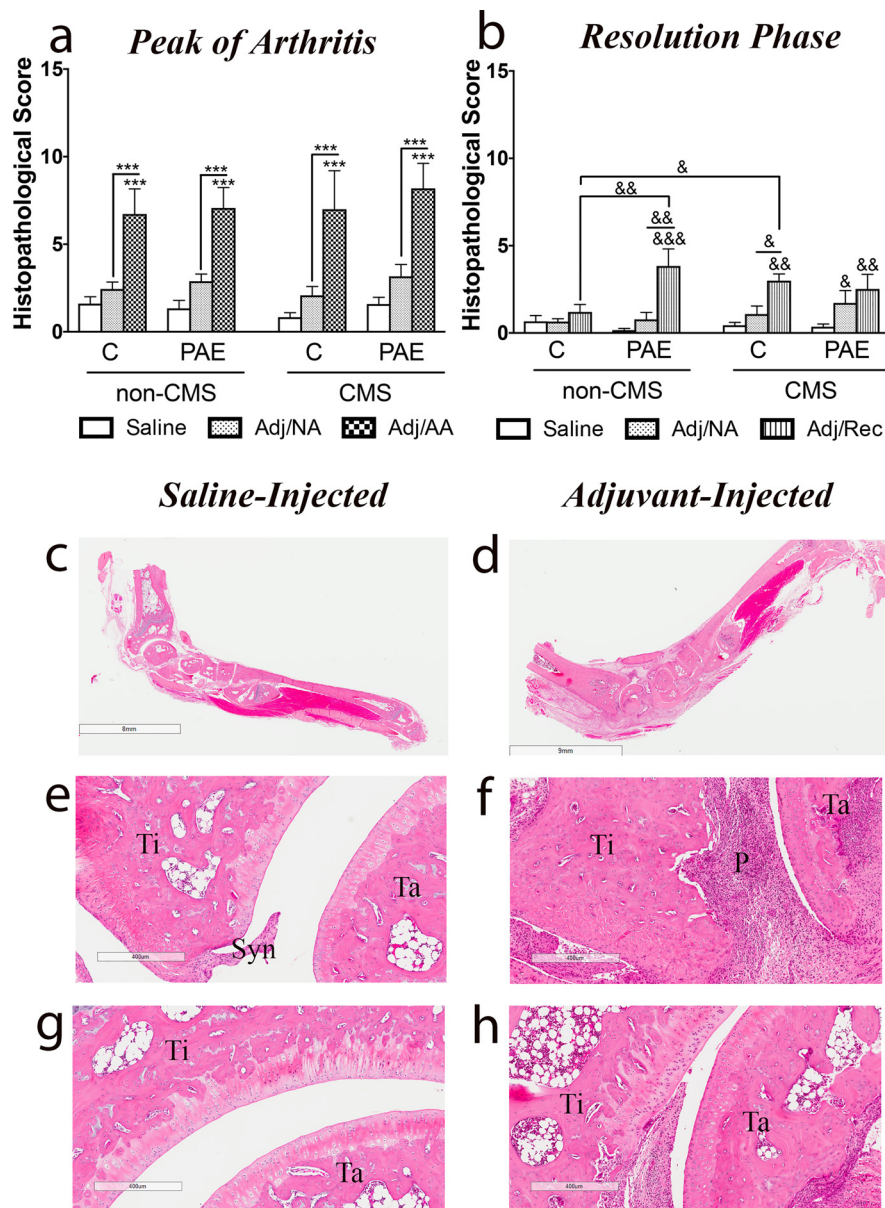


Figure 5. Histopathology by H&E staining of the tibiotarsal joint.

a,b: Total histopathological score (mean \pm SEM). a: At the peak of arthritis, the total histopathological score was higher in rats that developed arthritis (Adj/AA), compared to rats that failed to develop clinical signs of arthritis (Adj/NA) and saline-injected rats, across prenatal treatment and stress conditions. “***”: main effect of arthritis severity. b: During the resolution phase, in the non-CMS condition, the total histopathological score was higher in PAE rats that recovered from arthritis (Adj/Rec), compared to PAE rats that failed to develop clinical signs of arthritis (Adj/NA) and saline-injected rats. In addition, the total histopathological score was higher in PAE compared to C animals that recovered from arthritis (Adj/Rec). In the CMS condition, the total histopathological score was elevated in both C and PAE rats that recovered from arthritis (Adj/Rec), as compared to their saline-injected counterparts. In controls, this Adj/Rec group also had a higher score, as compared to rats that failed to develop clinical signs of arthritis (Adj/NA). By contrast, in PAE animals, the total histopathological score was elevated in rats that failed to develop clinical signs of arthritis (Adj/NA), as compared to their saline-injected counterparts. Finally, for animals that recovered from arthritis (Adj/Rec), the total histopathological score was higher in C rats exposed to stress (CMS), as compared to their non-stress counterparts. “&”: prenatal treatment \times stress condition \times arthritis severity interaction. c, e, g: Hind paw of a saline-injected rat. d, f, h: Hind paw of an adjuvant-injected rat. c–d: Scans showing overall H&E staining. e–h: Images showing the inferior edge of the tibia (e, f) and mid articulating cartilage between the tibia (Ti) and talus (Ta) (g, h). In e, g, articulating cartilage is visible at the ends of tibia and talus, and the joint space is clear, with the healthy edge of the synovium (Syn) visible. With active arthritis (f, h), the articulating cartilage and joint space is invaded by

internal inflammation, which is further supported by increased histopathological scores. Together, the drop in CBG in the face of increased corticosterone levels and increased tibiotarsal inflammation/damage is more characteristic of the profile seen at the peak of inflammation than that seen at recovery, suggesting that, from a physiological perspective, resolution is again impaired by PAE.

Utilization of female rats in this study enabled us to investigate the modulatory role of oestrogen in our PAE model. While clinical data are inconclusive as to the mechanism(s) underlying the increased risk of rheumatoid arthritis in females, hormonal factors have been shown to influence disease.³ Oestrogens have consistently been shown to have a protective role in terms of rheumatoid arthritis risk and/or severity during pregnancy³⁶ and oral contraceptive administration.⁴² Similarly, peak rheumatoid arthritis incidence occurs with the drop in sex hormone production at menopause.⁴³ In animal models, ovariectomy results in increased incidence and severity of experimentally-induced arthritis.⁴⁴ In the current model, at the peak of inflammation, oestradiol levels dropped with adjuvant injection (Adj/NA and Adj/AA), which is in line with chronic immune system activation disrupting the oestrus cycle and resulting in sustained dioestrus to prevent ovulation.⁴⁵ Not surprisingly, we did not find an association between oestrus stage and oestradiol levels, likely due to the high proportion of rats in dioestrus at termination (64%). At the recovery time point, however, our finding of highest oestradiol levels in adjuvant-injected rats that failed to develop arthritis (Adj/NA), across prenatal treatment and stress conditions, is unexpected. We propose that the increased oestradiol levels in this group may have had a protective effect against arthritis development. In support of this, oestradiol administration has been shown to dampen arthritis symptoms in animal models.⁴⁶

CRP, an acute-phase protein, is a commonly measured clinical marker of active inflammation and disease, including rheumatoid arthritis.⁴⁷ Here, CRP levels support earlier onset of active inflammation in PAE rats, as elevated CRP levels were detected on day 16 post-injection, prior to the onset of clinical signs of arthritis (Adj/NA) in both the non-stress and stress conditions. Of note, we have consistently identified alterations (or dysregulation) in CRP levels following PAE. For example, at birth, we reported decreased CRP levels in PAE rats,⁴⁸ with elevations in CRP detected by post-natal day 12.⁴⁹ During pregnancy in humans, we have

also identified an overall decrease in CRP levels with alcohol-consumption.⁵⁰ As CRP levels have also been reported to correlate with the severity and progression of a number of diseases,⁵¹ the consistent finding of alterations in CRP with alcohol-exposure/consumption suggests that this may be a cross-cutting feature of PAE and warrants further investigation.

Infiltration of immune cells into the synovium, subsequent inflammation, and bone and cartilage destruction are defining features of rheumatoid arthritis. Macrophages likely play a central pathogenic role, with other cell types such as neutrophils and T and B lymphocytes occupying more secondary roles.⁵² Specialized macrophage populations can be found in most tissues and organs, where they play key roles including removal of dead/dying cells and regulation of tissue homeostasis through sampling of the external environment.⁵³ Importantly, macrophage populations are particularly vulnerable to early-life insults, due to their wide-ranging distribution and their role in sampling the external environment.⁴⁰ In the present model, we found increased macrophage density within the synovium following recovery from arthritis (Adj/Rec). This finding, unique to PAE rats that were exposed to adolescent stress, suggests the possibility of differential arthritis pathophysiology in these animals. Specifically, joint damage in stress-exposed PAE rats may be more macrophage-dependent, potentially as a result of macrophage dysfunction. As macrophages were quantified using the cell surface glycoprotein CD163 (in order to avoid labelling synovial intimal fibroblasts), these CD163+ macrophages are generally classified as M2c, functioning to remove apoptotic cells⁵⁴ which is important for resolution from inflammation.⁵⁵ Therefore, the increased macrophage staining in animals exposed to PAE and adolescent stress may represent reparative processes within the tibiotarsal joint. Of note, however, detectable elevations in CD163+ macrophages were only found with active arthritis (Adj/AA) and in PAE animals exposed to stress that had recovered, from a clinical score perspective, from arthritis (Adj/Rec). As such, it stands to reason that despite no overt signs of arthritis, these rats are likely experiencing either continued inflammation at the cellular/tissue level and/or delays in the processes involved in the resolution from inflammation. Finally, this also highlights the importance of examining both external signs of arthritis (e.g. clinical scores), and including more in depth histopathophysiology (e.g. macrophage density and general signs of inflammation and bone and cartilage integrity). Thus,

panus (P). Statistical test (a,b): ANOVA; Significant *post hoc* comparisons: One symbol: $p < 0.05$; Two symbols: $p < 0.01$; Three symbols: $p < 0.001$; Saline: $n = 6$ /prenatal treatment/stress condition/time point; Adjuvant-injected: $n = 14$ /prenatal treatment/stress condition/time point. Three tibiotarsal sections were stained per rat and each section was scored by two independent researchers. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition; Saline: saline-injected; Adj/AA: Adjuvant-injected/Active arthritis (clinical score ≥ 1); Adj/NA: Adjuvant-injected/No clinical signs of arthritis; Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain); H&E: Haematoxylin and eosin.

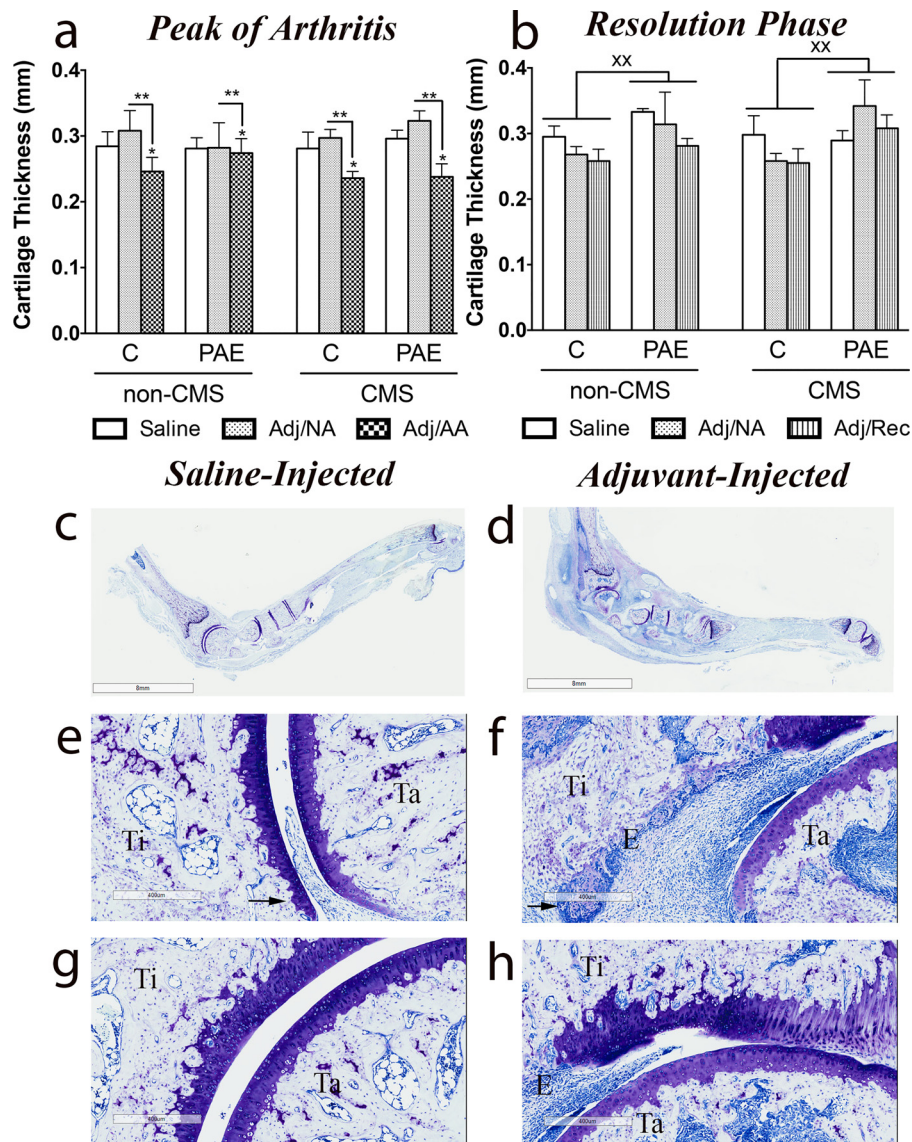


Figure 6. Cartilage integrity and thickness at the tibiotarsal joint.

a,b: Average cartilage thickness (mean \pm SEM) at the inferior edge of the tibia. a: At the peak of arthritis, cartilage thickness was decreased in rats that developed arthritis (Adj/AA), compared to rats that failed to develop signs of arthritis (Adj/NA) and saline-injected rats, across prenatal treatment and stress conditions. “**”: main effect of arthritis severity. b: During the resolution phase, PAE rats had increased cartilage thickness overall compared to controls, regardless of stress condition and arthritis severity. “ \times ”: main effect of prenatal treatment. c, e, g: Hind paw of a representative saline-injected rat. d, f, h: Hind paw of a representative adjuvant-injected rat. c,d: Scans of whole hind paws showing overall cartilage (toluidine blue) staining. e–h: Images showing the inferior edge of the tibia (e, f) and mid articulating cartilage between the tibia (Ti) and talus (Ta) (g, h). With saline-injection (e, g), the articulating cartilage (dark purple) is continuous at the distal tibia and proximal talus. With adjuvant injection (f, h), tibial cartilage is eroded (E) and the mid articulating cartilage shows thinning, decreased staining, and loss of the defined architecture seen in the saline condition. Arrows indicate the inferior edge of the tibia – the location at which cartilage thickness was measured. Statistical test (a,b): ANOVA; Significant *post hoc* comparisons: One symbol: $p < 0.05$; Two symbols: $p < 0.01$; Saline: $n = 6$ /prenatal treatment/stress condition/time point; Adjuvant-injected: $n = 14$ /prenatal treatment/stress condition/time point. One tibiotarsal section was stained per rat, with cartilage thickness measured by two independent researchers. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition; Saline: saline-injected; Adj/AA: Adjuvant-injected/Active arthritis (clinical score ≥ 1); Adj/NA: Adjuvant-injected/No clinical signs of arthritis; Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain).

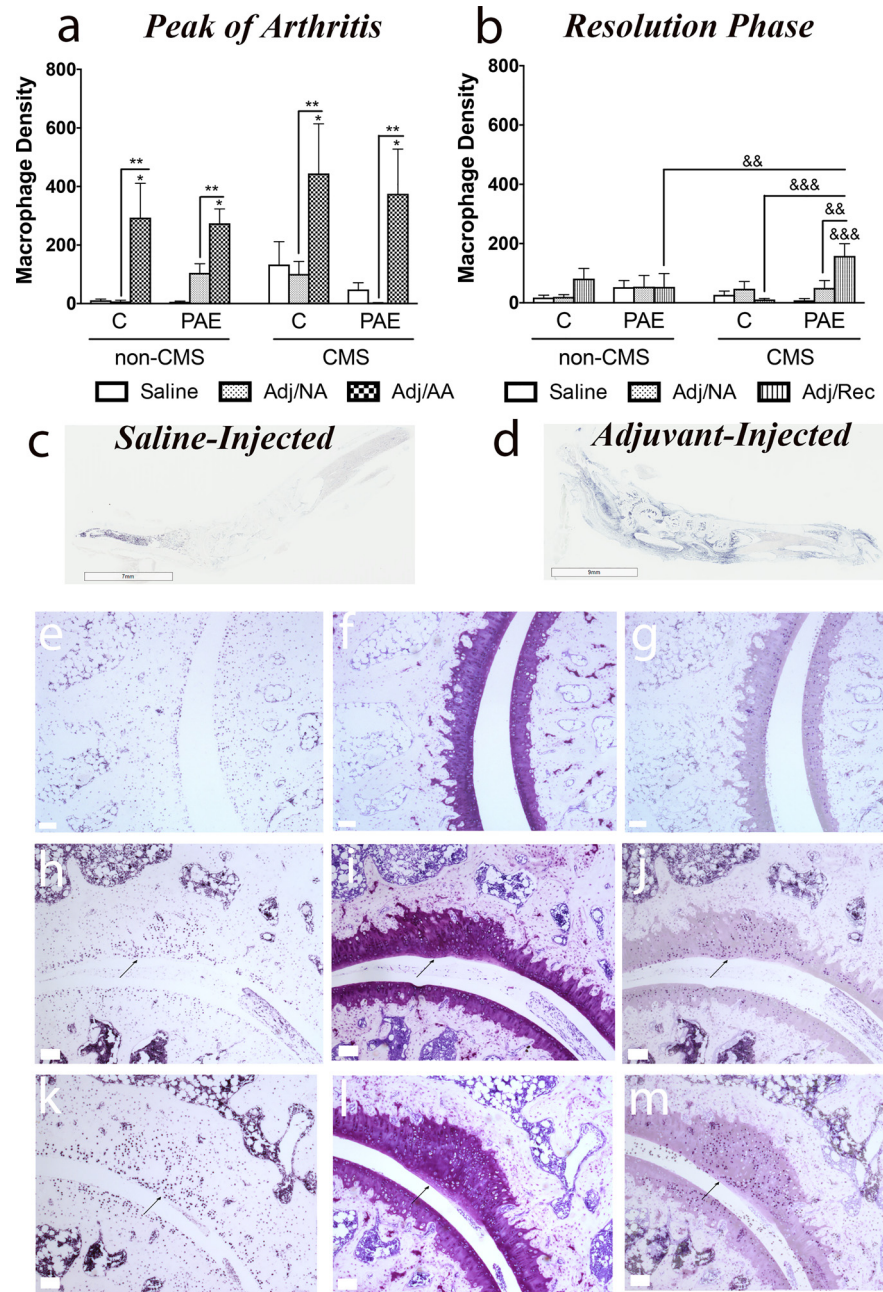


Figure 7. CD163⁺ macrophage density in the synovium and CD163⁺ chondrocyte staining in the cartilage. a,b: Average macrophage density (mean ± SEM) within the synovium. a: At the peak of arthritis, macrophage density was higher in rats that developed arthritis (Adj/AA), as compared to rats that failed to develop clinical signs of arthritis (Adj/NA) and saline-injected rats, across prenatal treatment and stress conditions. “***”: main effect of arthritis severity. b: During the resolution phase, in the CMS condition, PAE rats that recovered from arthritis (Adj/Rec) had higher macrophage density than both PAE rats that failed to develop clinical signs of arthritis (Adj/NA) and saline-injected PAE rats. In addition, macrophage density in PAE rats exposed to stress (CMS) that recovered from arthritis (Adj/Rec) was higher than all other groups of rats that recovered from arthritis (with the exception of C non-CMS rats). “&”: prenatal treatment × stress condition × arthritis severity interaction. c–d: Scans of whole hind paws showing overall CD163 staining. e, h, k: Mid tibia/talus CD163 staining; f, i, l: cartilage (toluidine blue) staining; g, j, m: overlay of CD163 and cartilage staining. Panels e–g: saline-injected rat; Panels h–j, k–m: rats with active arthritis, showing areas of increased cartilage thickness, populated by CD163⁺ chondrocytes (arrows). Statistical test (a,b): ANOVA; Significant *post hoc* comparisons: One symbol: *p* < 0.05; Two symbols: *p* < 0.01; Three symbols: *p* < 0.001; Saline: *n* = 6/prenatal treatment/stress condition/time point; Adjuvant-injected: *n* = 14/prenatal treatment/stress condition/time point. One tibiotarsal section was stained per rat, with

while we only detected impaired recovery from arthritis in PAE animals in the non-stress condition, this more in-depth description of the tibiotarsal joint suggests, perhaps, that with the combination of PAE and adolescent stress, inflammation and/or the long-lasting effects of inflammation, persist.

In addition to being expressed by mature tissue macrophages, CD163 is also expressed by phagocytic chondrocytes within the cartilage.⁵⁶ With arthritis progression, the percentage of CD163⁺ chondrocytes increases, resulting in enhanced migration and phagocytosis of cellular debris within the cartilage.⁵⁶ This is in line with our findings of tibial cartilage hyperplasia, which when overlaid with CD163⁺ staining, appears to be highly populated by CD163⁺ chondrocytes. We propose that our unexpected findings of increased cartilage thickness in the Adj/NA group at the peak of arthritis may be a result of increased phagocytotic activity of CD163⁺ chondrocytes, which is able to keep arthritis-associated inflammation in check. Comparatively, the decrease in cartilage thickness with active arthritis (Adj/AA) and the associated increase in total histopathological score, are likely a result of pannus invasion and destruction of the articular cartilage. Of note, at the recovery time point, we also showed overall increased cartilage thickness in PAE compared to control rats, which further points to heightened or long-lasting CD163⁺ activity within the articulating cartilage.

Finally, study limitations must also be considered. The inclusion of two experimental time points, the peak of arthritis and the resolution phase, allows terminal outcome measures to be considered at two time points during the disease course. However, it is important to note that direct comparisons between the two time points should be interpreted with caution. For example, comparing corticosterone levels in saline-injected control non-stress rats between the two time points shows differential baseline corticosterone levels: a mean of 301 ± 119 nM at the peak of arthritis compared to 73 ± 18 nM during the resolution phase. Yet there are underlying experimental differences that could account for this apparent discrepancy in baseline corticosterone levels. All animals were anesthetized in order to obtain accurate clinical scores, a confound that we must live with in this model. However, animals in the resolution phase were followed for a much longer time period, resulting in increased exposure to anaesthesia. Importantly, it has been shown that acute anaesthesia affects corticosterone levels⁵⁷ and that females are more susceptible to repeated anaesthesia.⁵⁸ Thus, differences in the amount of anaesthesia exposure make direct

comparisons between the two time points unreliable. Here, we selected isoflurane as the anaesthetic agent as it has generally been shown to have the least effect on a range of endocrine and metabolic factors,⁵⁹ and we included a saline-injected condition at both experimental time points in an attempt to account for the impact of anaesthesia on the outcome measures. Next, it is also worth noting that the combination of PAE and adolescent stress perhaps did not have the extensive synergistic effects, as originally hypothesized. For example, through examination of external signs of arthritis, PAE rats exposed to adolescent stress appeared to recover from arthritis more *quickly* than their non-stress counterparts. However, examination of the histopathology of the tibiotarsal joint revealed detectable damage in rats exposed to PAE and adolescent stress *even without* external signs of arthritis (Adj/NA group). In addition, the combination of PAE and adolescent stress resulted in increased macrophage density in rats that appeared to have recovered from arthritis (Adj/Rec). Taken together, these data indicate that measurement of external signs of inflammation alone (e.g. clinical scores) may not be sensitive enough to fully describe the pathophysiology of arthritis and that continued examination of other factors targeting the integrity of the tibiotarsal joints is warranted.

In summary, our findings highlight the immunomodulatory impact of adverse prenatal exposures in this chronic inflammatory model. The combination of PAE and adolescent stress resulted in independent and interactive effects at the level of hormonal, inflammatory and histopathological responses, which together suggest differential pathophysiology of disease. Further clinical investigation into incidence and prevalence of autoimmune disorders, including rheumatoid arthritis, in adults with FASD is urgently needed for a more complete understanding of the health problems in these individuals and for the development of appropriate and targeted treatment strategies.

Contributors

DM assisted with the CRP assays and analyses. LAH performed the CBG assays and contributed to writing of the CBG discussion. LE developed the CD163 protocol, completed the CD 163 assays, and assisted with animal procedures. WY contributed to the corticosterone and oestradiol assays and assisted with animal procedures. TSB and JW designed the experiment. TSB collected the data, performed the biological assays, completed the statistical analyses, and wrote the manuscript. TSB,

macrophage density measured by two independent researchers. C: control; PAE: prenatal alcohol exposure; Non-CMS: non-stress condition; CMS: stress condition; Saline: saline-injected; Adj/AA: Adjuvant-injected/Active arthritis (clinical score ≥ 1); Adj/NA: Adjuvant-injected/No clinical signs of arthritis; Adj/Rec: Adjuvant-injected/Recovered from arthritis (no clinical signs remain); Scale bars: 0.1 mm.

DM, LE, and LAH verified the underlying data. JW provided critical feedback in the revision of the manuscript. All authors read and approved the final manuscript.

Data sharing statement

Summary data are available in the paper and in supplementary materials. Raw data that support the findings of this study are available from the corresponding author upon request.

Declaration of interests

The authors have no competing interests to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2022.103876.

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