

The role of leukocyte caspase-1 activity in the development of epidural-related maternal fever: a single centre, observational, mechanistic cohort study.

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Abstract

Background: Epidural-related maternal fever (ERMF) has been reported in ~26% of labouring women. Explanatory mechanisms remain unclear. We hypothesized that ERMF is promoted by bupivacaine disrupting cytokine production/release from mononuclear leukocytes (MNF). We examined whether: (1) bupivacaine reduces caspase-1 activity and release of the anti-pyrogenic cytokine interleukin-1 receptor antagonist (IL-1ra); (2) bupivacaine is pro-inflammatory, through mitochondrial injury/interleukin-1 β (IL-1 β).

Methods: In labouring women, blood samples were obtained before/after epidural analgesia was implemented. Maternal temperature was recorded hourly for the first 4h of epidural analgesia. Time-matched samples/temperatures were obtained from labouring women without epidural analgesia, pregnant, non-labouring and non-pregnant women. The primary clinical outcome was change in maternal temperature over four hours after onset of siting epidural catheter/enrolment. The secondary clinical outcome was development of ERMF (temperature $\geq 38^{\circ}\text{C}$). The effect of bupivacaine/saline on apoptosis, caspase-1 activity, intracellular IL-1ra and plasma IL-1ra/IL-1 β ratio was quantified in MNF from labouring women or THP-1 monocytes (using flow cytometry, respirometry and/or ELISA).

Results: Maternal temperature increased by $0.06^{\circ}\text{C}/\text{h}$ (95%CI:0.03-0.09); $p=0.003$; $n=38$) following labour epidural placement. ERMF only occurred in women receiving epidural analgesia (5/38; 13.2%). Bupivacaine did not alter MNF or THP-1 apoptosis, compared to saline-control, but reduced caspase-1 activity by 11% (95%CI:5-17); $n=10$) in MNF from women in established labour. Bupivacaine increased intracellular MNF IL-1ra by 25% (95%CI:10-41; $p<0.001$; $n=10$), compared to saline-control. Epidural analgesia reduced plasma IL-1ra/IL-1 β ratio (mean reduction:14 (95%CI:7-30; $n=30$), compared to women without epidural analgesia.

Conclusion: Impaired release of anti-pyrogenic IL1-ra may explain ERMF mechanistically.

Immunomodulation by bupivacaine during labour may promote ERMF.

Introduction

Epidural-related maternal fever (ERMF) has been reported in up to 25% of women who receive epidural analgesia during labour.¹ Intrapartum fever is associated with more frequent obstetric interventions, ~30% higher rate of (unnecessary) antibiotic administration,² lower rates of spontaneous vaginal delivery and excess neonatal morbidity,³ However, the molecular mechanism(s) underlying ERMF remain unclear.⁴ The maternal compartment may be the primary source of inflammation,^{5,6} since an infectious placental aetiology is absent in the majority of women with ERMF.⁷

Fever requires the systemic release of pyrogenic cytokines, chiefly interleukin IL-6 and IL-1 β .⁸ Fever is inhibited by the anti-pyrogenic cytokine interleukin-1 receptor antagonist (IL-1ra),⁸ which directly inhibits the pro-inflammatory effects of IL-1 β through binding to the interleukin-1 receptor. Modulation of IL-1 β and/or IL-1ra by local anaesthetic agents used routinely for obstetric analgesia is a plausible mechanism underlying ERMF.¹ Local anaesthetics exert anti- and pro-inflammatory effects in a dose-dependent, tissue specific manner.⁹ Bupivacaine may promote inflammation through mitochondrial damage, triggered by uncoupling mitochondrial oxidative phosphorylation and inhibiting mitochondrial respiratory chain complexes.¹⁰⁻¹² The resultant increase in reactive oxygen species (ROS) production is similar to that driven by acute systemic inflammation, which repurposes mitochondrial function from ATP synthesis to production of ROS.¹³ Metabolic reprogramming and ROS generation activate the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome,^{14 15} one of several that promote the maturation and secretion of interleukin IL-1 β and IL-18 and subsequent downstream release of fever-inducing cytokines. Caspase-1 is essential for the formation of the NLRP3 complex and IL-1 β production in leukocytes,¹⁵ but also for triggering cell death and regulating the release of IL-1ra through unconventional protein secretion.¹⁶

ERMF is associated with increases in maternal and umbilical cord serum inflammatory cytokines.⁶ Therefore, we hypothesized that ERMF is promoted by bupivacaine disrupting cytokine production/release from mononuclear leukocytes (MNF) during established labour by altering caspase-1 and NLRP3 activity. To test this hypothesis, we first examined whether clinically relevant concentrations of bupivacaine altered leukocyte metabolism (using a THP-1, human monocyte cell line). We further examined whether regulation of cytokine release in mononuclear leukocytes by bupivacaine was specific to women in established labour, by comparing leukocyte biology in 4 populations: labouring women with and without epidural analgesia, non-labouring pregnant women prior to elective caesarean delivery and non-pregnant women undergoing other elective surgery.

METHODS

Patients

This study was conducted at University College London Hospitals NHS Trust (ISRCTN11281491) from October 2014 to May 2016. Ethical approval was obtained from the South Central Oxford NRES Committee (reference: 14/SC/1160). All women recruited into the study provided written consent to participate in this study. Four populations of women were recruited into this single centre, observational, mechanistic cohort study: (1) women in established labour who requested epidural analgesia; (2) women in established labour who did not receive epidural analgesia; (3) non-labouring pregnant women prior to elective caesarean delivery; (4) non-pregnant women of child-bearing age undergoing (non-obstetric) elective surgery (Supplementary Figure 1). Inclusion criteria for labouring women were: ≥ 18 years old, ≥ 37 weeks of gestation, singleton pregnancy and confirmed to be in established labour (>3 cm dilatation, with regular uterine contractions). Exclusion criteria were: pre-eclampsia, hypertensive disorder of pregnancy, pre-existing immune dysfunction, known infection and/or pyrexia, current antibiotic prescription and/or non-steroidal anti-inflammatory use. For the other two (non-labour) groups, we recruited age-matched women who satisfied the same exclusion criteria.

Women in labour

Labouring women on the labour ward were recruited into the epidural analgesia arm of the study, and labouring women in the birthing centre (midwife led without availability of epidural analgesia) were recruited into the non-epidural analgesia arm of the study. All women had access to Entonox and intramuscular diamorphine. Women requesting epidural analgesia while on the birthing centre are transferred to labour ward prior to epidural catheter insertion. Because pregnancy is a transient, but physiological, period of hyper-cortisolism

with maintained diurnal variation of plasma cortisol levels,¹⁷ recruitment was standardised by the time of data collection (morning) to minimise any confounding influences on immune function. Women were recruited on weekday mornings when women meeting the inclusion criteria were identified and research fellow and laboratory researchers were available to recruit patients, collect data and perform experiments. Patients recruited to the pregnant non-labouring and non-pregnant arms of the study were approached on the morning of their elective caesarean delivery or elective non-obstetric surgery respectively.

Clinical Procedures

Epidural catheter insertion technique and analgesia regimen

Prior to epidural catheter insertion, labouring women in our institution upon request can receive Entonox (oxygen and nitrous oxide) and a single dose of intramuscular diamorphine 7.5 mg. Following verbal and written consent to participate in the study a 16 gauge intravenous catheter was placed. An epidural catheter was sited in the mid-lumbar epidural space using a loss-of-resistance to saline technique, and following negative aspiration, an initial dose of 15 mL 0.1% bupivacaine / 2 mcg/mL fentanyl (low dose mixture) in 3 divided doses of 5 mL, 5 minutes apart (with regular vital sign assessment) and women were subsequently connected to a patient controlled epidural analgesia pump (PCEA; Smiths Medical, UK) administering an 8mL bolus with 20 minute lockout. The PCEA solution was the same solution as that used to initiate analgesia.

Primary clinical outcome.

The primary clinical outcome was change in maternal oral temperature over four hours after onset of siting of epidural catheter, or for labouring women who did not receive epidural analgesia, 4h after enrolment (Figure 1A). A previous study has reported that maternal temperature rises after epidural analgesia over a similar period of time.¹⁸ During labour, oral temperature correlates most closely with intrauterine temperature and is recommended for routine detection of maternal pyrexia in labour.¹⁹ We therefore measured oral temperature (Welch Allyn Suretemp plus 690 thermometer, Aylesbury, UK; calibration accuracy $\pm 0.1^{\circ}\text{C}$) hourly in each labour group (epidural and no epidural analgesia) immediately following written consent and prior to epidural catheter placement in the epidural analgesia group. Subsequent temperatures were measured at hourly intervals for four hours after the first blood sample and temperature reading were acquired.

Secondary clinical outcomes

We recorded episodes of ERMF (maternal oral temperature $\geq 38^{\circ}\text{C}$) during labour up to delivery, requirement for sepsis or fever evaluation and indications for antibiotic use. Patients, attending clinicians and critical care staff were blinded to the patients' laboratory data. All laboratory staff were masked to patients' details including temperature.

Laboratory Procedures

Isolation of primary leukocytes

Patient leukocytes were isolated using the density gradient technique (Ficoll Plus, BD Biosciences, Oxford, UK) within 20 minutes of venous whole blood samples (10ml) being collected, as described previously.²⁰ Residual erythrocytes were eliminated by hypotonic buffer lysis. Mononuclear cells (monocytes, lymphocytes) were assessed.

Flow cytometry

Flow cytometry gating strategy and antibodies used are shown in Supplementary Methods. All flow cytometry measurements were conducted on $\geq 10,000$ cells (FACScalibur). Cell debris and dead cells were excluded from the analysis based on scatter characteristics and propidium iodide fluorescence. Lymphocyte and monocyte subsets were identified by CD45⁺CD3⁺, CD45⁺CD19⁺ and CD45⁺CD14⁺ staining. Isotype-matched controls or omitting secondary antibodies were used throughout to control for non-specific binding of antibodies.²⁰ Analysis was undertaken using Kaluza and/or FlowJo (version 10) software.

Bupivacaine.

We used 10 μ M bupivacaine based on plasma concentrations measured after low-dose epidural infusion in labour,²¹ and transversus abdominal plane blocks following caesarian section.²² This concentration is below levels associated with systemic effects²³ and toxicity ($\geq 17 \mu$ M).²⁴

Laboratory Experiments

Five mechanistic questions were addressed in the laboratory, which are summarised in Table 1.

Experiment 1: Does bupivacaine cause cell death and hence inflammation through mitochondrial dysfunction?

We quantified changes in mitochondrial respiration and glycolysis, using the Seahorse XF96 analyser by measuring oxygen consumption and extracellular acidification rates, as described previously (see Supplementary data for full details).²⁰ We assessed whether bupivacaine and/or circulating inflammatory mediators in pooled plasma obtained from women in established labour triggered mitochondrial dysfunction in the THP-1 human monocytic

leukemic cell line (InvivoGen; San Diego, California, USA), which is commonly used for the study of inflammasome activation as they express high levels of NLRP3, inflammasome adaptor protein apoptosis-associated Speck-like protein containing CARD (ASC) and pro-caspase-1.²⁵ We also examined whether the absence of NLRP3 inflammasome in THP1-NLRP3 deficient cells (THP-1-NLRP3def; InvivoGen; San Diego, California, USA) altered any effects evoked by bupivacaine.²⁶

Metabolic measurements were made in the presence/absence of bupivacaine. At least 6 measures were made per genotype/serum sample. As a total of 92 wells were available for cell plating, some experimental (genotype/treatment) combinations were assayed in 7, rather than 6, wells. All measurements were corrected to total protein content for each well (BCA protein assay, Pierce Biotechnology). Glycolysis was quantified concomitantly by measuring extracellular acidification rate. Spare respiratory capacity, which serves as a measure of the extra capacity available within cells to respond rapidly to acute increases in metabolic demand, was calculated as the difference between ionophore-induced maximal, and basal rate, of oxygen consumption. Further details are provided in Supplementary material (methods).

Experiment 2: Does bupivacaine cause cell death and hence inflammation through increased apoptosis?

We used samples obtained from women who did and did not receive epidural analgesia at the beginning of the time period over which maternal temperature was measured (Figure 1A). *Ex vivo* monocytes and lymphocytes from the same women in established labour were incubated with bupivacaine for the 4h period (Figure 1B), to mimic the amount of time these cells were exposed to bupivacaine *in vivo*. Annexin V staining was undertaken, as described previously (see Supplementary Methods for flow cytometry density plots).²⁰ At least 5×10^5 mononuclear

cells were washed in phosphate buffered saline and then resuspended in Annexin buffer (100 μ L) containing Annexin V FITC and/or propidium iodide (PI) or buffer alone. Early apoptosis was quantified as the percentage of the whole population of cells expressing annexin-V FITC (PI-negative).

Experiment 3. Does pregnancy and/or established labour alter caspase-1 activation in presence/absence of bupivacaine?

To address this question, mononuclear cells were obtained from 3 groups of women: 1. those in established labour (epidural or nonepidural analgesia); 2. non-pregnant women prior to elective surgery; 3. pregnant women not in labour. Using the 4h time period shown in Figure 1B, we quantified caspase-1 activity by flow cytometry pre and post epidural, or after 4h *ex vivo* incubation, using the FLICA (FLuorescent Inhibitor of CAspases) technique (ImmunoChemistry Technologies, ABD Serotec, Oxford, UK). This *in vitro* assay exploits the properties of a cell permeant fluorescent inhibitor probe FAM-YVAD-FMK to label established caspase-1 enzyme in living cells.²⁰ Green fluorescence serves as a direct measure of the established caspase-1 enzyme activity present in the cell at the time the reagent was added. All cells were fixed and analysed within 1h. Nigericin and/or gramicidin (both 10 μ M), which activate caspase-1 and inflammasome formation, were used as a positive control in each experiment.

Experiment 4: Does bupivacaine alter IL-1ra release in leukocytes obtained from women in established labour?

Monocytes and lymphocytes obtained from women in established labour were incubated with 10 μ M bupivacaine (Figure 1B) to establish its' impact on the release of intracellular IL1-ra. Following fixation for CD14/CD3/CD19 surface staining, cells were permeabilized

(Cytotfix/Cytoperm, BD Biosciences, UK) to enable intracellular staining for IL-1ra expression (AF-280-NA; R and D Systems, Abingdon, UK).

Experiment 5: Does bupivacaine alter plasma IL-1ra/IL-1 β ratio in women in established labour?

We quantified plasma IL-1ra and IL-1 β levels in paired plasma samples obtained before the initiation of epidural analgesia, and 4h later; time-matched samples were also obtained from women who received non-epidural analgesia. IL-1ra/IL-1 β ratios were calculated for each sample, which reflects the balance between pro-inflammatory IL-1 β and anti-inflammatory IL-1ra components of the NLRP3 inflammasome.²⁷ We used a solid phase enzyme-linked human IL-1ra/IL-1F3 immunoassay that measures IL-1ra in duplicate plasma samples (100 μ l each), referenced to an *E. coli*-derived recombinant human IL-1ra (R&D Systems, Abingdon, UK). Intra-assay precision has a coefficient of variation (CV) 3.7-7.3%; inter-assay CV is 6.7-11% (standard curve is shown in Supplementary Figure 2). We measured plasma IL-1 β cells by colorimetric enzyme assay. HEK-Blue™ IL-1 β cells (Invivogen, Toulouse, France) were used to detect bioactive IL-1 β , which binds to the IL-1R surface receptor. Subsequent activation of the NF- κ B/AP-1-inducible Secretion of Embryonic Alkaline Phosphatase (SEAP) reporter gene leads to the production of SEAP. Levels of SEAP were quantified by a colorimetric enzyme assay (QUANTI-Blue™, Invivogen, Toulouse, France) referenced to a known concentration of recombinant human IL-1 β .

Statistical methods

For continuous data, normality of distribution was assessed (Kolmogorov–Smirnov test). As indicated, data were analysed with paired t-test, one-way or two way ANOVA (with Tukey-Kramer post-hoc testing). Mean (SD) or median values (interquartile range) are presented,

unless stated otherwise. The correlation between bupivacaine dose and change in caspase-1 activity was also evaluated by linear regression. All reported p values are two-sided. Significance was accepted at p values ≤ 0.05 . Statistical analyses were performed using NCSS 11 (Kaysville, UT, USA).

Sample size calculation

The sample size was calculated to take into account both clinical and mechanistic experiments, in accord with design principles for these types of studies.²⁸ We hypothesised that early biological changes and temperature increase are plausibly linked with ERMF and may be detected by the laboratory experiments. ERMF may occur in up to 26% labouring women after epidural analgesia.¹ With a predicted incidence of fever of at most 3% in the non-epidural analgesia group, a sample size of ≥ 33 women would be required to detect ERMF ($\alpha=0.05$, $1-\beta=80\%$), which was increased to 38 women to cater for a potential dropout rate $\sim 25\%$. This sample size calculation is similar to a study in which temperature increase was measured over 4h in 40 labouring women who received epidural analgesia, compared to 13 women who received parental opioids ($\alpha=0.05$, $1-\beta=80\%$). From our previous published work, detecting biologically significant changes in caspase-1 activity would require ≥ 7 paired samples per experimental group.²⁰

RESULTS

Patient characteristics

Women in the epidural and nonepidural groups shared similar characteristics. (Table 2).

There was no significant difference between stage of labour (cervical dilatation) at the time of recruitment of labouring women with/without epidural analgesia. Several women did not have blood drawn for serial paired analyses (Supplementary Figure 1), including age/weight matched pregnant women not in established labour and women presenting for elective gynaecological surgery (Supplementary Table 1).

Primary clinical outcome: serial maternal temperature measurements during labour.

All labouring women were afebrile and without clinical evidence of infection at the beginning of the study. None had experienced premature rupture of membranes. A single dose of intramuscular diamorphine was administered to 8/38 women prior to epidural analgesia. In the epidural group, women received mean (SD) bupivacaine dose 52 (20) mg. Four hours after baseline measurement of oral temperature, the mean temperature was greater than baseline in the epidural, but not non-epidural, group (Figure 1C). No women developed ERMF within this initial 4h study period. Women in the non-epidural analgesia group all received nitrous oxide and oxygen for variable durations; 4/15 also received a single dose of intramuscular diamorphine 7.5 mg during labour.

Secondary clinical outcomes

Epidural-related maternal fever was recorded in 5/38 (13.2%) women prior to delivery. All five women were assessed for suspected development of sepsis during labour. By contrast, in labouring women who did not receive epidural analgesia, we did not record a single episode of fever (maternal temperature $\geq 38^{\circ}\text{C}$ prior to delivery) or any sepsis workup during labour.

In the non-epidural analgesia group, prophylactic antibiotics were administered to 1/15 (6.6%) prior to delivery (for confirmed group B *Streptococcus*), compared to 4/38 (10.5%) women who had epidural analgesia. 4/15 women (26.7%) converted to epidural analgesia after the initial (four hour) study period during which they received opiate analgesia.

Laboratory measures

Experiment 1: Does bupivacaine cause cell death and hence inflammation through mitochondrial dysfunction?

Spare respiratory capacity, a measure of metabolic reserve, was similar in the presence/absence of 10 μ M bupivacaine (Figure 2A; Supplementary Figures 3,4). Similarly, lipopolysaccharide increased glycolysis ($p < 0.001$), but no difference was observed between control versus bupivacaine treated samples (Figure 2B; Supplementary Figures 3,4).

Experiment 2: Does bupivacaine cause cell death and hence inflammation through apoptosis?

Incubation of MNF for 4h with bupivacaine did not increase apoptosis in either CD3+ lymphocytes (mean difference:6% (95%CI:-4 to 18); $p=0.18$) or CD14+ monocytes (mean difference:2% (95%CI:-5 to 8); $p=0.55$) obtained from women in established labour (Figure 2C-D). We observed similar apoptosis in lymphocytes obtained 4h after epidural analgesia (Supplementary Figure 5).

Experiment 3. Does pregnancy and/or established labour alter caspase-1 activation in presence/absence of bupivacaine?

We next assessed whether bupivacaine (10 μ M) altered caspase-1 activity in mononuclear leukocytes obtained before epidural analgesia was instituted in labouring women ($n=10$ paired samples; Figure 3A-E). Mononuclear leukocyte cells incubated for 4h with

bupivacaine reduced caspase-1 activity, in CD3⁺ lymphocytes (Figure 3A, B) by 14% (95%CI:5-16%) After epidural analgesia was instituted, reduced caspase-1 activity correlated with the dose of bupivacaine that five women required for analgesia over the 4 h period ($R^2=0.88$, $p=0.02$; Figure 3C). A decline in caspase-1 activity was also observed in CD14⁺ monocytes (Supplementary Figure 6).

To test whether the effect of bupivacaine on caspase-1 activity in leukocytes was dependent on the acute inflammation associated with established labour, we obtained mononuclear leukocytes from six age-and gestation-matched women not in established labour in whom blood samples were obtained before elective caesarean section. As in established labour, bupivacaine reduced caspase-1 activity in CD3⁺ lymphocytes incubated for 4h with serum obtained from women in established labour prior to epidural catheter insertion ($p<0.001$; left panel, Figure 3D). By contrast, MNF caspase-1 activity was unchanged when CD3⁺ lymphocytes were incubated with non-labouring pregnant serum from elective caesarean delivery patients ($p=0.14$; right panel; Figure 3D). Similarly, caspase-1 activity was unchanged after bupivacaine treatment for 4 h in samples obtained from (non-pregnant) women prior to elective laparoscopic gynaecological surgery ($n=9$; $p=0.68$; Figure 3E; Supplementary Figure 7).

Experiment 4: Does bupivacaine alter IL-1ra release in leukocytes obtained from women in established labour?

Since we found that bupivacaine consistently reduced caspase-1 activity in labouring women, we reasoned that the reduction in caspase-1 activity after bupivacaine may limit the release of the anti-pyrogenic cytokine IL-1ra and therefore promote the likelihood of ERMF.

Intracellular IL-1ra protein levels were increased when leukocytes were incubated with bupivacaine for 4h, compared to control ($p<0.001$; $n=10$ labouring women; Figure 4A-C).

Experiment 5: Does bupivacaine alter plasma IL-1ra/IL-1 β ratio in women in established labour?

Samples from women in established labour showed that plasma IL-1ra/IL-1 β ratio only declined in labouring women who received epidural analgesia (p=0.017; Figure 4D). Plasma IL-1 β did not differ between analgesic regimes (Supplementary Figure 8).

Discussion

The main clinical findings of our study are consistent with a similar prospective study where women receiving epidural analgesia had a significant increase in temperature after 4h of analgesia, a trend that was absent in women who received parenteral opioids only for analgesia.¹⁸ Moreover, the ERMF rate was similar in our study to the ~20% incidence identified from 22 studies by systematic review.¹

Our mechanistic data revealed three main findings. First, bupivacaine reduces caspase-1 activity in circulating mononuclear leukocytes obtained from women in labour. Second, the reduction in caspase-1 activity in mononuclear cells incubated with clinically relevant concentrations of bupivacaine was mimicked only by adding serum obtained from women in established labour. Third, the reduction in caspase-1 activity was paralleled by reduced release and higher intracellular levels of IL-1ra, indicating that bupivacaine appears to impair release of this anti-pyrogenic cytokine. Taken together, these data suggest that bupivacaine, at clinically relevant concentrations, may disrupt pivotal elements of inflammasome-related biology in leukocytes during established labour, thereby increasing the risk of ERMF. Our data offer an explanation that reconciles the apparently paradoxical observation in many ERMF studies that have reported a similar rise in pro-inflammatory

cytokines in both women receiving epidural and non-epidural analgesia.⁵ In humans, a ratio of IL-1Ra/IL-1 β >100 appears to correlate with functional inhibition of the biological effects of IL-1 β .^{29,30} It therefore seems likely that the rise in pro-pyrogenic cytokines may only result in fever when release of IL-1Ra, an endogenous anti-pyrogenic antagonist, is reduced.

Our finding that ERMF may be promoted by impaired IL-1ra release (as summarised in Supplementary Figure 9) is supported by laboratory and clinical work implicating this cytokine in fever, including studies performed in pregnancy. First, recombinant IL-1ra decreases foetal systemic inflammation generated by the release of IL-1 β through chorioamnionitis in sheep.³¹ Similarly, in rats, placental inflammation stimulated by microbial challenge is alleviated by the co-administration of IL-1ra.³² Second, familial Mediterranean fever that is refractory to colchicine prophylaxis has been successfully treated with anakinra, an interleukin-1-blocking agent.³³ Third, the variability in incidence of ERMF may be accounted for, in part, by IL-1ra gene polymorphisms. Two common variants located upstream of IL1RN, the gene encoding IL-1ra, are the strongest known genetic determinants of increased soluble IL-1ra protein concentration. The frequency of IL-1ra-raising alleles is ~30% for the rs6743376 variant, and ~50% for rs1542176.³⁴

We did not find any metabolic/mitochondrial evidence for bupivacaine being injurious in either a cell line model or mononuclear cells obtained from women in labour. Taken together, these data suggest that bupivacaine is not directly injurious to mononuclear cells *in vitro* or *ex-vivo* at clinically relevant concentrations. Many preceding studies exploring the toxic effects of bupivacaine have employed far higher concentrations (frequently in the millimolar range) that exceed clinically safe levels.^{35,36} We used a monocyte cell line for three principle reasons. First, attaining sufficient numbers of monocytes from patients for respirometry is challenging with a limited volume of patient blood. Using this established THP-1 model in a standardized manner enables a reproducible

picture of acute changes in immune-metabolism triggered by acute inflammation.²⁵ Second, THP-1 cells have high IL-1ra activity. Third, in monocytes caspase-1 activation is uncoupled from pathogen-associated molecular pattern recognition (in contrast to macrophages which require TLR ligands and subsequent adenosine triphosphate stimulus).³⁷

Strengths of this study include the early measurement of temperature during established labour, which were coupled with time-matched *ex-vivo* studies. This approach enabled us to study leukocyte biology over the same time period, during which cellular changes may develop that predispose/contribute to developing fever. The clinical study was adequately powered to detect ERMF, as suggested by our literature review.¹ Similarly, the sample sizes in laboratory investigations were adequate based on our previous work detailing biologically relevant changes in caspase-1 activity,²⁰ and in accord with recommendations on devising translational studies in humans.²⁸ Real-time, rapid laboratory assays of leukocytes masked to clinical details were obtained from both labouring women before epidural catheters were sited and those who initially chose not to have epidural analgesia. Because pregnancy is a transient, but physiological, period of hyper-cortisolism with maintained diurnal variation of plasma cortisol levels,¹⁷ laboratory assays were standardized by the time of data collection (morning) to minimise any confounding influences on immune function.

The main limitations for the secondary clinical and mechanistic outcome studies were the 26.7% conversion rate from non-epidural to epidural analgesia (after the 4h temperature study period) and the unpredictable availability of samples for the timely processing of real-time laboratory assays in a significant number of women. Furthermore, patient refusal to participate (chiefly in the non-epidural analgesia group) also limited the number of paired samples. We acknowledge that labouring women received epidural analgesia in a physically separate location (labour ward) with obstetrician-guided management, in contrast to women in the birthing centre where care was led by midwives.

Mechanistically, assessing circulating leukocytes was an accessible first step, but the impact of bupivacaine on caspase-1-mediated cytokine release from leukocytes that accumulate in gestational tissues and the placenta may be more substantial during established labour.³⁸ Finally, we appreciate modes of analgesia may be difficult to compare owing to the fact that women choosing epidural analgesia may experience more painful, longer and / or more complicated labour compared to women selecting other less invasive analgesic modalities. Furthermore, in murine models, acute infection produces pain by directly activating sensory neurons that modulate inflammation.³⁹

In summary, the mechanism underlying ERMF may involve bupivacaine impairing the release of the anti-pyrogenic cytokine interleukin-1 receptor antagonist (IL-1ra) from circulating leukocytes by reducing activation of caspase-1. While our preliminary cytokine-based data require clinical validation in larger cohorts of women, a more detailed mechanistic understanding of the inflammatory biology associated with epidural analgesia during labour may help refine obstetric management of fever through the development of a novel, precision medicine approach.

Authors contributions: GLA, AGDA, JS designed the experiments. GLA, AGDA, JS, SP performed the data analysis. SPa SPh AR SS PS ALD GLA, EPIFEVER Investigators collected samples and organised clinical recruitment. The manuscript was drafted by GLA, PS, AGDA and revised following critical review by all authors.

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Disclosures

ALD: director of Magnus Growth, part of Magnus Life Science, which is aiming to take to market a novel treatment for fetal growth restriction. GLA: Editor, British Journal of Anaesthesia; editorial advisory board for Intensive Care Medicine Experimental; consultancy work for GlaxoSmithKline, unrelated to this work.

Table 1. Summary of mechanistic experiments.

Experiment	Question	Outcome assessed (assay)	Sample source	Number/timing of sample(s)	Sample type(s)	Clinical comparison	Ex-vivo experimental comparisons	n	Stats
1	Does bupivacaine cause mitochondrial dysfunction (alone or in presence of inflammation)?	Oxidative phosphorylation and glycolysis (cellular respirometry)	Cell line	4h incubation	THP1 null, NLRP3 -/-	N/A	Metabolism over time: effect of genotype of cell on LPS ± bupivacaine OR control [PBS] ± bupivacaine	≥6	2-way ANOVA (genotype v. bupivacaine/control)
2	Does bupivacaine induce apoptosis/cell death in MNF leukocytes obtained from women in established labour?	Apoptosis (annexin-V), cell death (propidium iodide)	¹ . Established labour, with epidural. ² . Established labour, no epidural	2 samples: before, and 4h after, insertion of epidural	CD3 ⁺ , CD14 ⁺	¹ . Pre-epidural v. 4h post-epidural	² . Pre-epidural OR non-epidural sample incubated with control [PBS] OR bupivacaine for 4h	9	^{1,2} Paired t-test
3	Does bupivacaine increase caspase-1 activation in MNF leukocytes obtained from women in established labour?	Caspase-1 activity (FLICA, flow cytometry)	¹ . Established labour. ² . Non-pregnant women (child-bearing age). ³ . Non-labouring, pregnant women.	2 samples: before, and 4h after	CD3 ⁺ , CD14 ⁺	Pre-epidural v. 4h post-epidural	^{1,2,3} . Sample incubated with control [PBS] OR bupivacaine for 4h	¹ 10 ² 6 ³ 9	¹ 2-way repeated measures ANOVA (time x analgesia) ^{2,3} Paired t-test
4	Does bupivacaine alter intracellular IL-1ra protein levels in MNF leukocytes obtained from women in established labour?	Intracellular IL-1ra protein (flow cytometry)	Established labour.	Single sample before insertion of epidural	CD3 ⁺ , CD14 ⁺ , CD19 ⁺	N/A	control [PBS] vs. bupivacaine	10	Unpaired t-test
5	Does epidural bupivacaine reduce plasma IL-1ra/IL1β ratio?	IL-1ra protein (ELISA)	¹ . Established labour, with epidural. ² . Established labour, no epidural	2 samples: before, and 4h after, insertion of epidural/alternative analgesia	plasma	Baseline v. 4h after	N/A	^{1,3} 3 ^{2,1} 0 ⁰ 0	2-way ANOVA (time x mode of analgesia)

PBS- Phosphate buffered saline [control]; MNF=mononuclear fraction; CD3 = lymphocyte;

CD14=monocyte, CD19=B Lymphocyte. LPS- lipopolysaccharide. FLICA- Fluorochrome Inhibitor of Caspases.

^{1, 2, 3} denote experimental group denoted in each row.

Figure Legends

Figure 1. Temperature measurements during early phase of epidural and non-epidural analgesia.

A. Clinical protocol for measuring temperature during established labour in women receiving epidural or non-epidural analgesia.

B. Parallel experimental protocol for quantification of caspase-1 activity in clinical samples measured *ex-vivo* in leukocytes obtained from labouring women, by FLICA ((FLUorescent Inhibitor of CASpases) assay).

C. Serial oral temperature measures over 4h. Median values (white line), 25-75th centiles shown in box plots (whiskers:10-90 centiles). $p < 0.001$ derived from repeated measures ANOVA (time x type of analgesia).

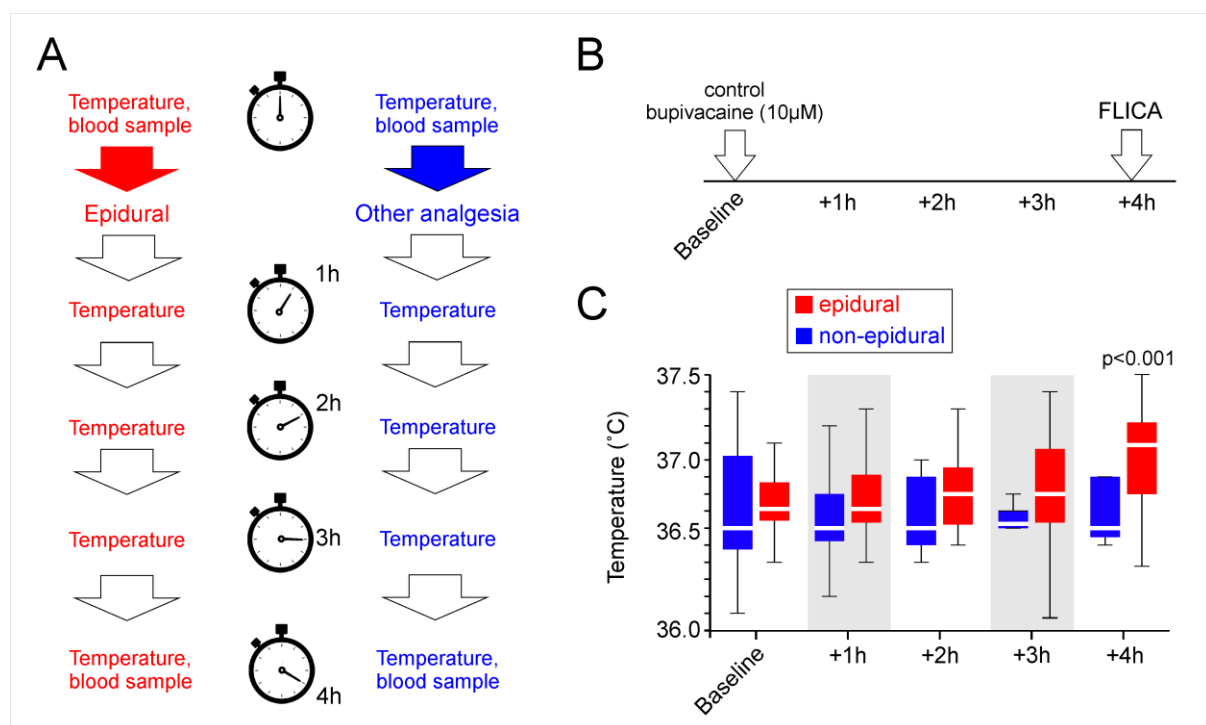


Figure 2. Cellular respirometry in THP-1 cells exposed to bupivacaine. (Experiments 1, 2).

- A. Spare respiratory capacity in THP-1 cells exposed to lipopolysaccharide (100ng.ml⁻¹) for 4h in presence/absence of bupivacaine (10μM) or phosphate-buffered saline (control). Median (IQR) values shown (Mann Whitney U test; Experiment 1).
- B. Extracellular acidification rate, an indirect measure of glycolysis. LPS increased glycolysis (p<0.001), but no difference was observed between control (phosphate-buffered saline) v. bupivacaine treated samples. p<0.001 denotes difference between lipopolysaccharide vs. control samples (ANOVA: LPS/control x local anaesthetic). No difference was observed between control v. bupivacaine samples (post-hoc Tukey-Kramer test; Experiment 1)
- C. Apoptosis was unchanged (p=0.18) in CD3⁺ lymphocytes obtained just before epidural analgesia after 4h incubation with bupivacaine or control (phosphate-buffered saline). (n=9 women; Mann Whitney U test; Experiment 2).
- D. Apoptosis was unchanged (p=0.55) in CD14⁺ monocytes cells obtained just before epidural analgesia after 4h incubation with bupivacaine or control (phosphate-buffered saline). (n=9 women; Mann Whitney U test; Experiment 2).

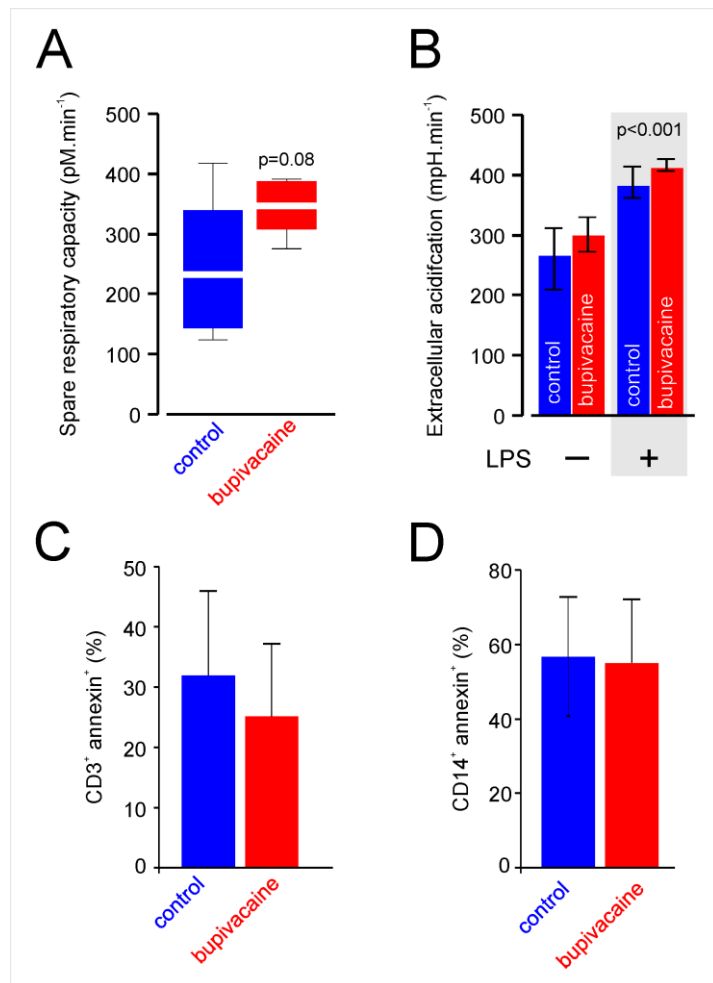


Figure 3. Bupivacaine and caspase-1 activity in circulating leukocytes (Experiment 3).

- A. Histogram showing caspase-1 fluorescence in CD3⁺ leukocytes obtained prior to epidural analgesia, with bupivacaine (10 μ M) or control (phosphate-buffered saline). Grey histogram is unstained cell population (n=10 women in established labour).
- B. Summary data for CD3⁺ caspase-1 fluorescence (% cells with caspase-1 activity), compared by paired t-test (p=0.007).
- C. Significantly correlated reduction in % CD3⁺ cells with caspase-1 activity as function of cumulative bupivacaine dose received by labouring women during 4h study period (R²=0.88; p=0.02; n=5 women who received epidural analgesia). Grey shaded area denotes decline from pre-epidural analgesia caspase-1 activity. Red lines denote 95% confidence intervals.
- D. Effect of control serum from C-section donor, or serum from women in established labour obtained prior to epidural catheter insertion, on caspase-1 activity in CD3⁺ T cells obtained from 6 age/gestation matched women prior to elective caesarean section, in presence of bupivacaine or control (phosphate-buffered saline) for 4h. Blue/red boxes summarise protocol to assess impact of labour serum on C-section MNF caspase-1 activity, in absence/presence of bupivacaine (10 μ M). ‘Established labour’ serum increased caspase-1 activity (p<0.001), compared to control serum from C-section donor; fewer CD3⁺ cells displayed caspase-1 activity when co-incubated with bupivacaine, compared to control (phosphate-buffered saline) for 4h (ANOVA: serum source x bupivacaine/saline control).

E. Population data for caspase-1 activity in freshly isolated MNF obtained from non-pregnant women presenting for elective surgery, in presence of bupivacaine or control (phosphate-buffered saline) for 4h. (n=9 female surgical patients, compared by paired t-test). Data are shown as median (white bar) with 25-75th centiles (box edges; whiskers 10-90 centiles).

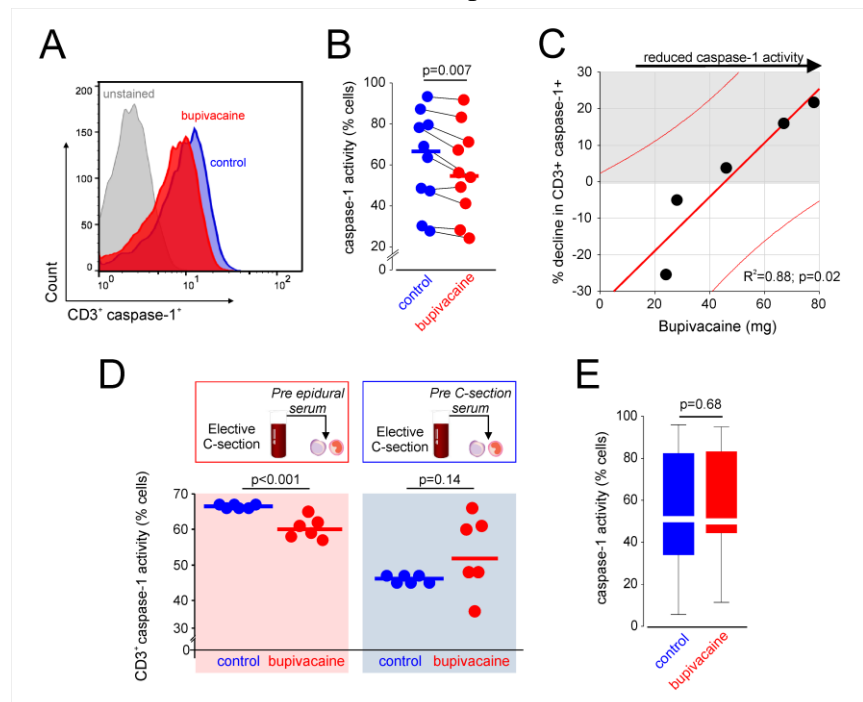
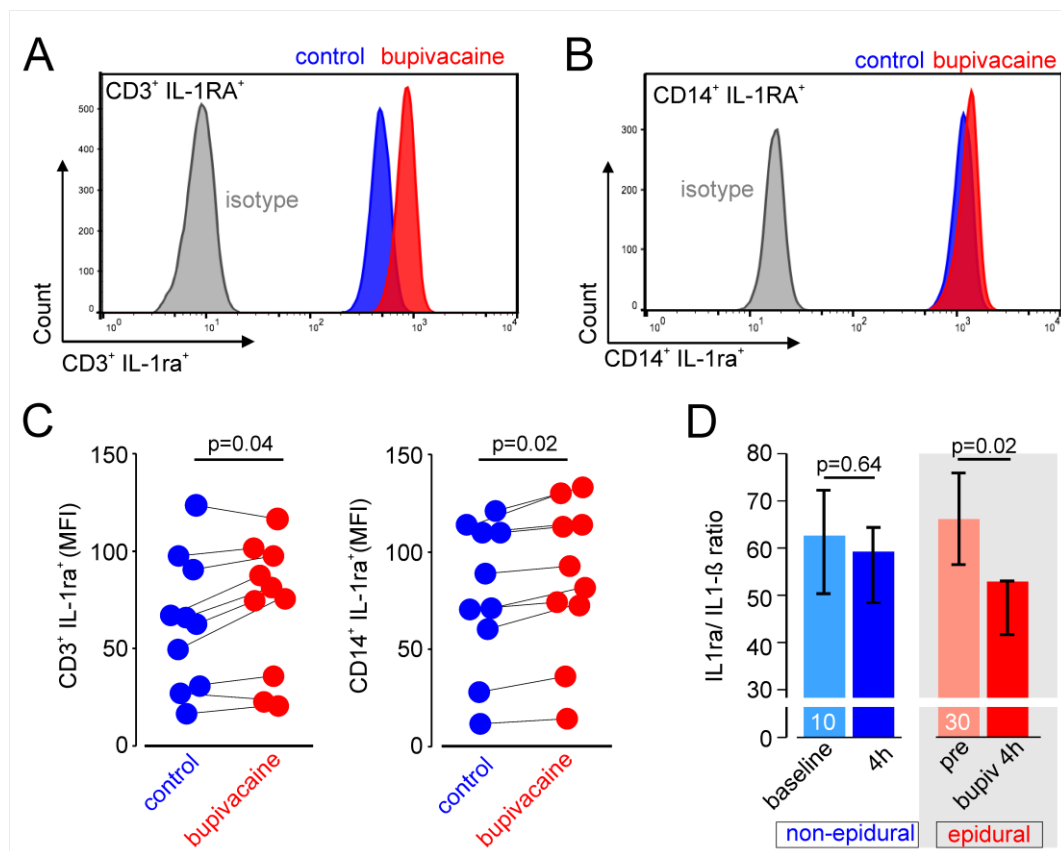


Figure 4. IL-1ra protein levels in epidural/non-epidural analgesia (Experiments 4, 5).

- A. Histogram showing CD3⁺ intracellular IL-1ra expression following 4h exposure to bupivacaine or control (phosphate-buffered saline; Experiment 4)
- B. Histogram showing CD14⁺ intracellular IL-1ra expression following 4h exposure to bupivacaine or control (phosphate-buffered saline; Experiment 4).
- C. Summary data for CD3⁺ IL-1ra⁺ and CD14⁺ IL-1ra⁺ (intracellular) expression after 4h incubation with bupivacaine or control (phosphate-buffered saline). Each joined dot represents the same donor (n=10), compared by paired t-test. (Experiment 4).
- D. IL-1ra/IL-1 β ratio over 4h in women who received epidural (n=30), or non-epidural analgesia (n=10). Mean (95%CI) values shown. p=0.02 derived from two-way ANOVA (analgesia x time point; Experiment 5).



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Supplementary online material

The role of leukocyte caspase-1 activity in the development of epidural-related maternal fever: a single centre, observational, mechanistic cohort study.

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Supplementary Methods

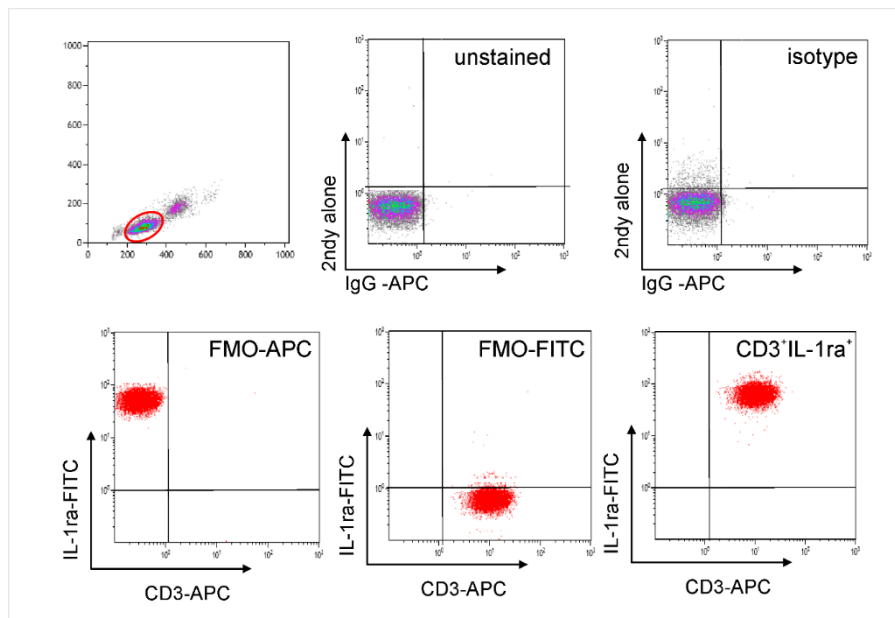
Flow cytometry antibodies.

APC- *Allophycocyanin*

Antibody-fluorochrome	Clone	Isotype	Source
CD14-APC	TUK4	Mouse IgG2a	Miltenyi Biotec Ltd
CD3-APC	BW264/56	mouse IgG2ak	Miltenyi Biotec Ltd
CD-19-APC	LT19	Mouse IgM	Miltenyi Biotec Ltd
IL-1ra	AF-280-NA	Unconjugated IgG polyclonal goat	R and D Systems

Flow cytometry gating.

FMO- fluorescence minus one. APC- *Allophycocyanin*. FITC- Fluorescein isothiocyanate



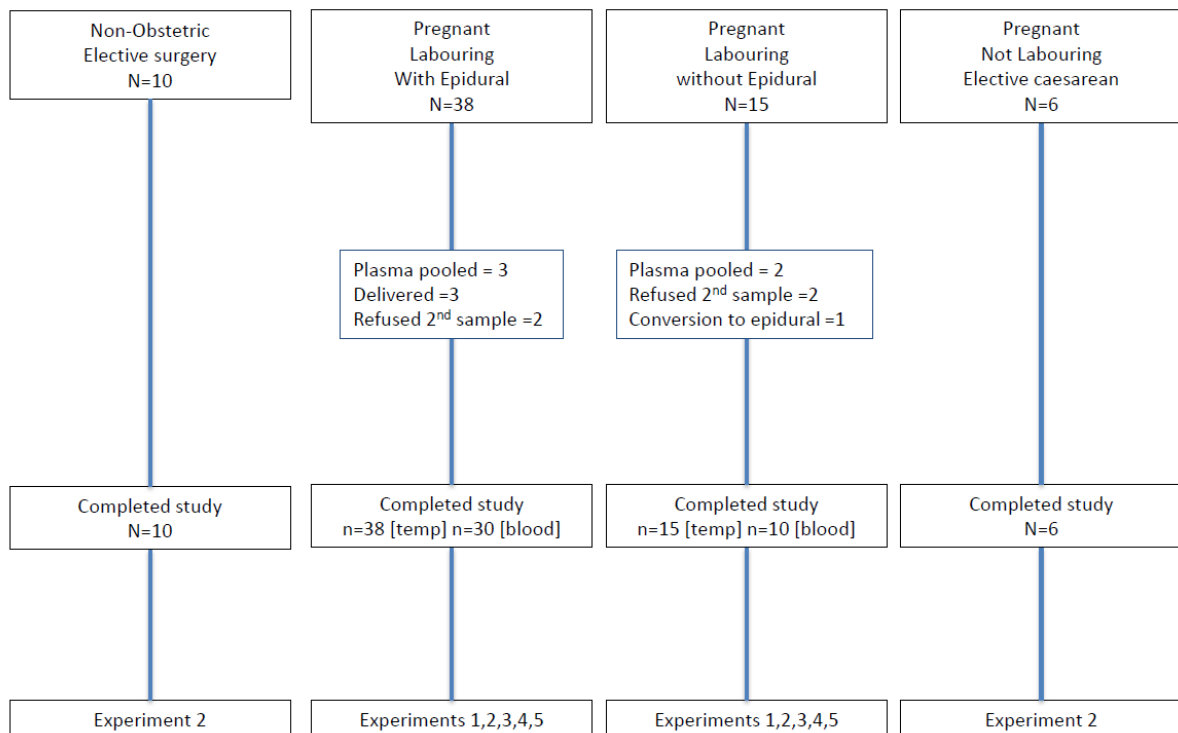
Mitochondrial function/cellular respirometry [experiment 1]

Oxygen consumption was measured at baseline and after addition of the uncoupling agent FCCP (2 μ m; carbonyl cyanide prifluoromethoxyphenylhydrazone) to quantify maximal oxygen consumption. Non-mitochondrial respiration was quantified by inhibiting mitochondrial respiration through addition of rotenone (1 μ M) and antimycin A (1 μ M). Mitochondrial respiration coupled towards ATP production was measured by the fall in oxygen consumption following addition of oligomycin (0.2 μ g.ml⁻¹), an inhibitor of ATP synthase. The difference between the maximum *respiratory capacity* and basal *respiratory capacity* is referred to as the *spare respiratory capacity*.

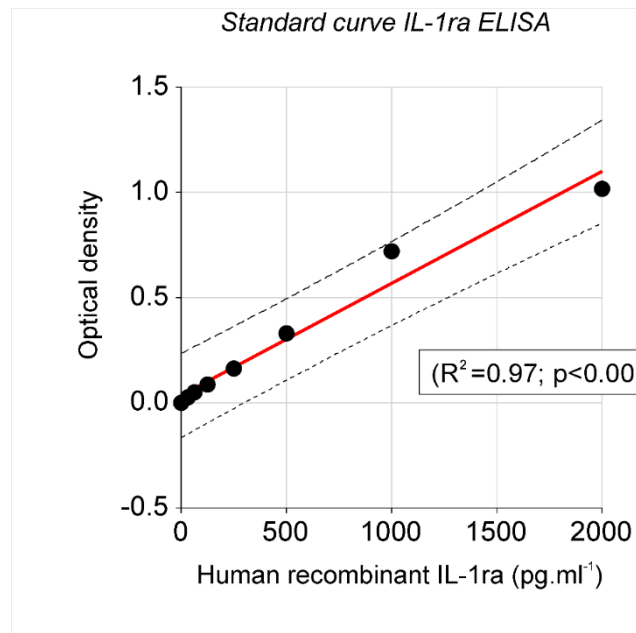
Supplementary Table 1. Age and weights of pregnant and non-pregnant participants. Mean age (range) and weight (SD) shown.

	Age (years)	Weight (kg)
Epidural	34 (23-42)	62 (10)
Non-epidural	32 (24-38)	67 (20)
Pre C-section	33 (23-42)	64 (14)
Preoperative [non-pregnant]	34 (20-41)	not collected

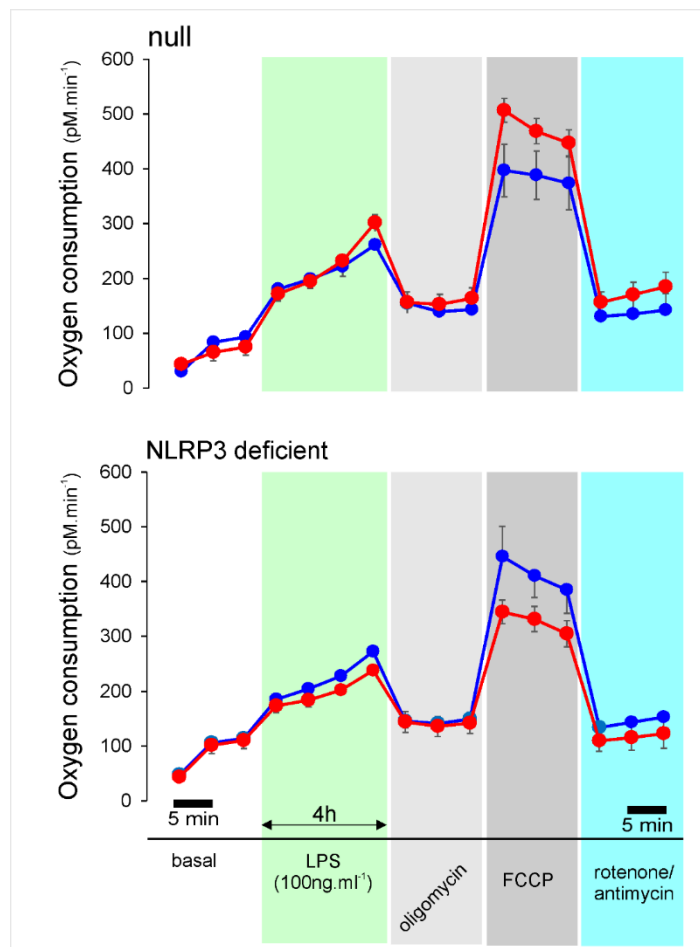
Supplementary Figure 1: Patient flow diagram



Supplementary Figure 2: Experiment 5- Standard curve for IL-1ra ELISA assay.

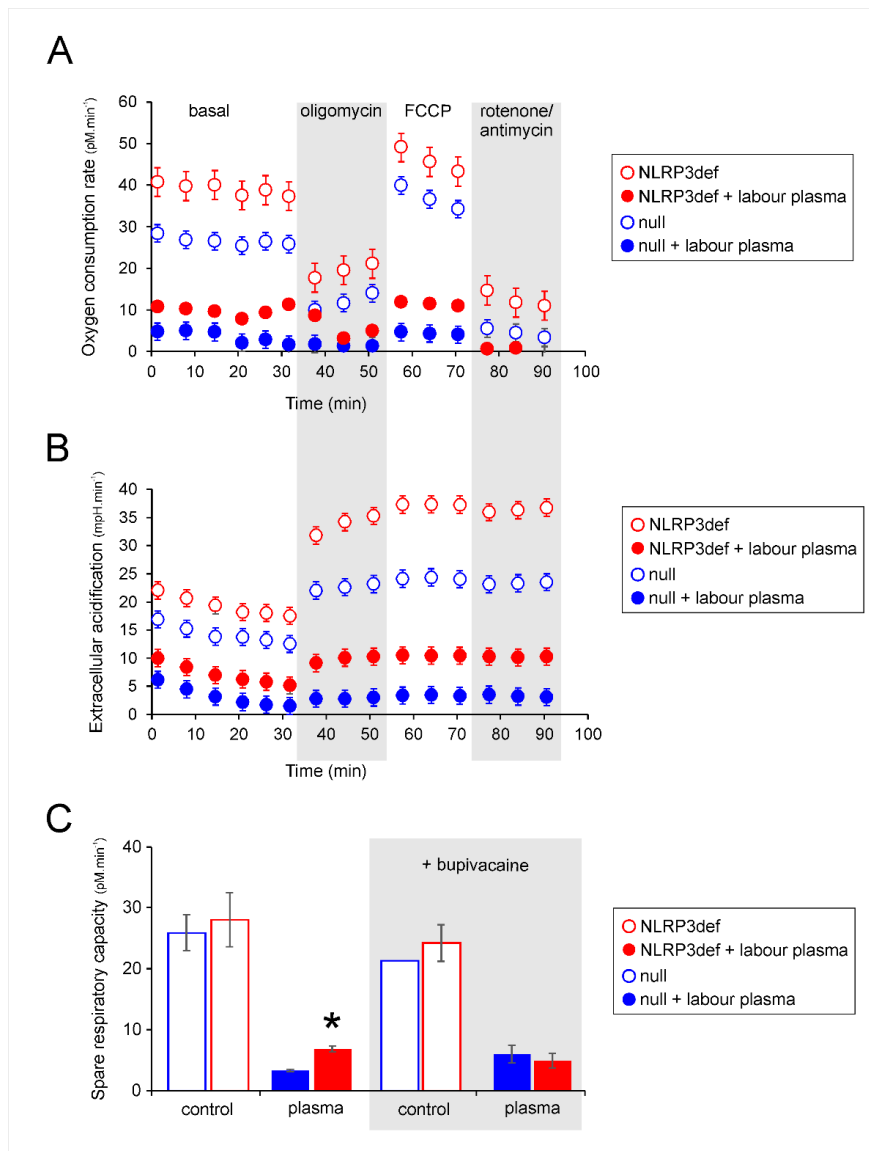


Supplementary Figure 3: Experiment 1- raw respirometry data showing metabolic effect of LPS in THP-1 monocyte-like human cell line in presence (red)/absence (blue) of bupivacaine. Data mean [95%CI]

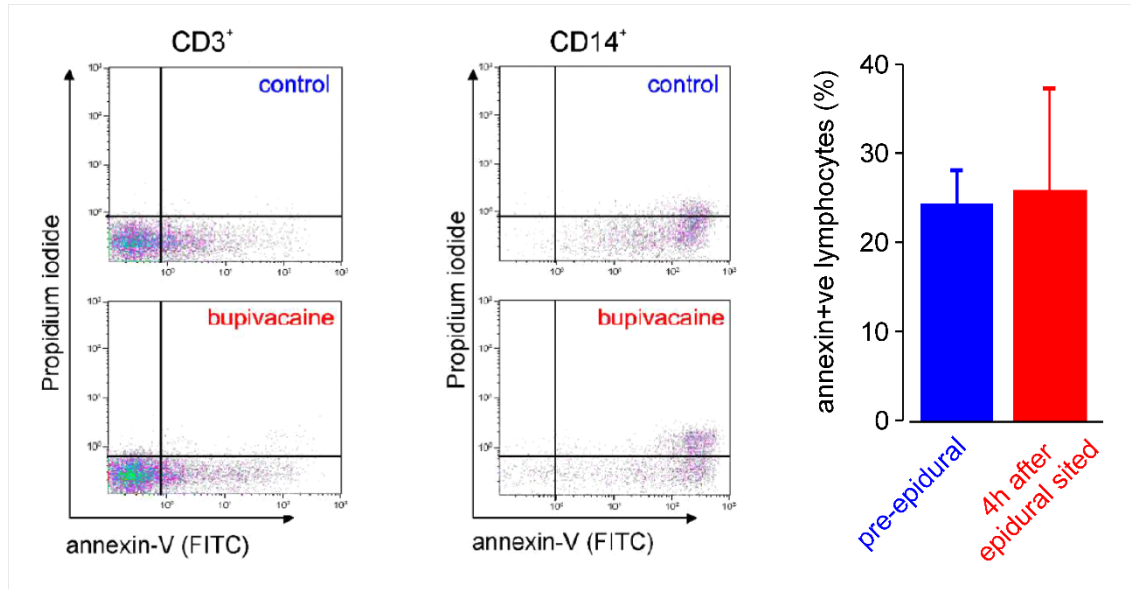


Supplementary Figure 4: Experiment 1- raw respirometry data showing effect of plasma from women in active labour on bioenergetics of THP-1 null and NLRP3 deficient monocyte-like human cell line in presence/absence of bupivacaine.

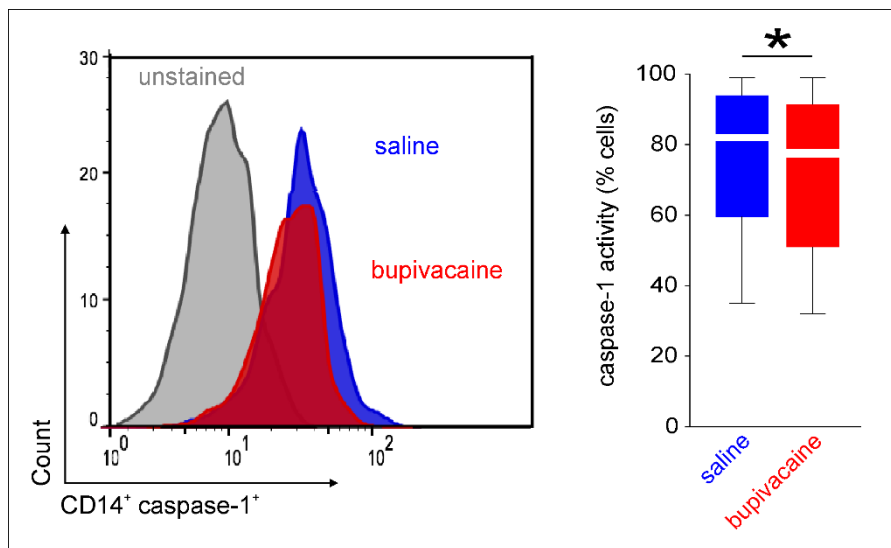
A. oxidative phosphorylation and B. glycolysis (ECAR) in THP-1 null and NLRP3 deficient monocyte-like human cell line in presence/absence of bupivacaine and plasma from active labour. C. Spare respiratory capacity, a measure of metabolic reserve which reflects the ability of cells to cope with acute bioenergetic stress triggered by inflammatory stimuli, was similar in the presence/absence of 10 μ M bupivacaine. The difference between the maximum *respiratory capacity* and basal *respiratory capacity* is referred to as the *spare respiratory capacity*. Data mean [SD]; asterisk denotes $p < 0.05$, post-hoc Tukey test.



Supplementary Figure 5: Experiment 2- Density plots showing apoptosis in CD3+ and CD14+ caspase-1 activity after bupivacaine treatment for 4h, compared to control (phosphate-buffered saline). Graph shows apoptosis was similar in CD3+ lymphocytes (mean (SD)) before, and 4h after, epidural bupivacaine was administered (mean dose 52mg (SD:20)).

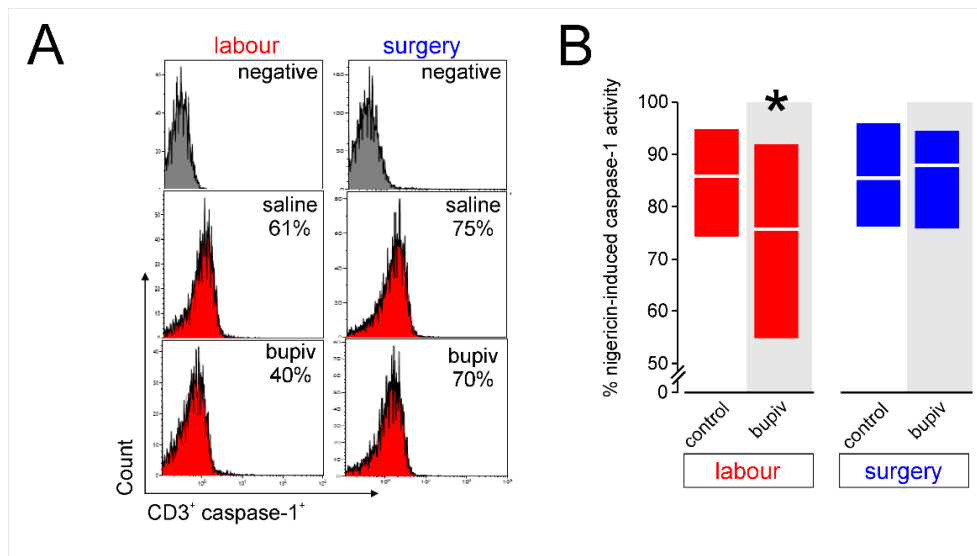


Supplementary Figure 6: Experiment 3- CD14+ monocyte caspase-1 activity obtained from labouring women, after incubation with bupivacaine (10µM) or control (phosphate buffered saline). Data are median[IQR]. Asterisk denotes p<0.05 [paired t-test].



Supplementary Figure 7: Experiment 3- effect of bupivacaine on caspase-1 activity in leukocytes obtained from women not in labour.

Mononuclear leukocytes were obtained from age-matched, non-pregnant women of child-bearing age presenting for elective surgery. In contrast to the effect of bupivacaine on caspase-1 activity in leukocytes obtained from labouring women, caspase-1 activity was similar after incubation with bupivacaine (10 μ M) or control (phosphate buffered saline) for 4h in samples obtained from (non-pregnant) women prior to elective surgery (n=9; p=0.68). Data are median[IQR] expressed as % nigericin response. Asterisk denotes p<0.05; ANOVA/post-hoc Tukey test.



Supplementary Figure 8: Experiment 5- Plasma levels of IL-1 β before and 4h after epidural

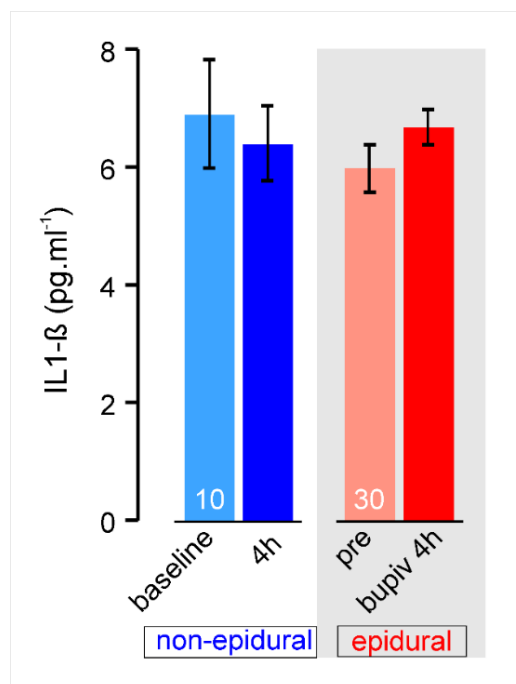
(n=30), or non-epidural (opioid) analgesia

(n=10), in labouring women. Mean (95%CI)

values shown. There was no difference

between IL-1 β in non-epidural versus epidural

samples (p=0.26, ANOVA)



Supplementary Figure 9: Proposed role of IL1-ra in ERMF.

In women receiving non-epidural forms of analgesia, activation of the inflammasome via activated caspase-1 releases IL-1 β , and subsequent release of cytokines such as IL-6, the fever-promoting features of which are attenuated by the parallel release of the anti-pyrogenic cytokine IL1-ra. In women receiving epidural analgesia with bupivacaine, inhibition of caspase-1 activity reduces the release of the anti-pyrogenic cytokine IL1-ra. Loss of this anti-fever mechanism increases the chances of ERMF.

IL-1 β . IL-6: interleukin-6. IL-1 β : interleukin-1 beta. Casp1: active caspase-1

