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The effect of sex, menstrual cycle phase and oral contraceptive use on intestinal permeability and *ex-vivo* monocyte TNFα release following treatment with lipopolysaccharide and hyperthermia

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ABSTRACT

Purpose: Investigate the impact of sex, menstrual cycle phase and oral contraceptive use on intestinal permeability and *ex-vivo* tumour necrosis factor alpha (TNFα) release following treatment with lipopolysaccharide (LPS) and hyperthermia.
Methods: Twenty-seven participants (9 men, 9 eumenorrheic women (MC) and 9 women taking an oral contraceptive pill (OC)) completed three trials. Men were tested on 3 occasions over 6 weeks; MC during early-follicular, ovulation, and mid-luteal phases; OC during the pill and pill-free phase. Intestinal permeability was assessed following a 4-hour dual sugar absorption test (lactulose: rhamnose). Venous blood was collected each trial and stimulated with 100 $\mu g \cdot mL^{-1}$ LPS before incubation at 37 °C and 40 °C and analysed for TNFα via ELISA.
Results: L:R ratio was higher in OC than MC (+0.003, $p = 0.061$) and men (+0.005, $p = 0.007$). Men had higher TNFα responses than both MC (+53 %, $p = 0.004$) and OC (+61 %, $p = 0.003$). TNFα release was greater at 40 °C than 37 °C (+23 %, $p < 0.001$).
Conclusions: Men present with lower resting intestinal barrier permeability relative to women regardless of OC use and displayed greater monocyte TNFα release following whole blood treatment with LPS and hyperthermia. Oral contraceptive users had highest intestinal permeability however, neither permeability or TNFα release were impacted by the pill cycle. Although no statistical effect was seen in the menstrual cycle, intestinal permeability and TNFα release were more variable across the phases.

1. Introduction

The gastrointestinal (GI) tract is involved in multiple inflammatory and autoimmune disorders, including Crohn's disease, ulcerative colitis, and Celiac disease. These disorders are characterized by altered GI barrier permeability, which causes downstream activation of immune responses and the subsequent production of pro-inflammatory cytokines [33]. GI barrier function is also altered by circulating sex hormones, with increases in GI symptoms such as abdominal pain and diarrhoea reported by women during periods of low oestrogen concentrations (follicular phase, menstruation) [3,7,15]. Oral contraceptive (OC) users report a worsening of GI symptoms, including abdominal pain, diarrhoea and indigestion during the pill-free weeks when not taking the synthetic hormones [19]. As compared to men, there is also a fourfold greater preponderance of GI disorders (coeliac disease, Crohn's disease, and irritable bowel syndrome) in women [24]; Lovell and Ford 2012). Together, these data suggest that low concentrations of female sex hormones can influence the GI barrier and intestinal permeability,

Abbreviations: CI, confidence interval; EIA, Enzyme Immunoassay; ELISA, Enzyme-linked Immunosorbent Assay; GI, gastrointestinal; IBS, irritable bowel syndrome; L:R, ratio, Lactulose Rhamnose ratio; LPS, lipopolysaccharide; MC, menstrual cycle; OC, oral contraceptive; TAE, Tris-acetate-EDTA; TNFα, tumour necrosis factor alpha.

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which may contribute to changes in downstream immune and inflammatory responses [26].

Among inflammatory cells, monocytes play a crucial role in innate immunity and are important when countering viral and bacterial infections via the production and release of cytokines such as tumour necrosis factor alpha (TNFα). Monocyte activity is sex-dependent [13] with lipopolysaccharide (LPS) induced TNFα release shown to be much higher in men compared to women in most [1,2,5,8], but not all [34] studies. Greater release of TNFα in men following LPS exposure might also explain why men exhibit higher mortality rates from septic shock (Lefevre et al., 2012) and exertional heat stroke [14]. Divergent GI barrier functionality and differences in immune activation and inflammatory processes could therefore be expected in men, as compared to women at different phases of the menstrual cycle, and/or women who use hormonal contraceptives.

On this basis, the purpose of the present study was to quantify sex-specific differences in GI barrier function at rest, and assess whether GI permeability was augmented by oral contraceptive use throughout the menstrual cycle. To examine the effects of sex, menstrual cycle phase, and oral contraceptive use on monocyte function (TNFα release), we conducted *ex-vivo* experiments in which venous whole blood was treated with LPS and incubated under 'normal' (37 °C) and 'hyperthermic' (40 °C) conditions. Experiments were conducted in men, and in women during the early-follicular, ovulation, and mid-luteal phase of the menstrual cycle. To ensure the full range of applicable comparisons, oral contraceptive users were also tested during the pill-free and pill phases.

2. Materials and methods

2.1. Participant characteristics

Twenty-seven healthy, physically active participants completed the study: nine men, nine eumenorrheic women (natural menstrual cycle - MC) and nine women taking a monophasic combined oral contraceptive pill (OC) (Table 1). Fig. 1 provides details on the recruitment process. The study was approved by the University of Chichester Ethics committee (Protocol Number 1819_46) and all participants provided written informed consent and completed health history questionnaires before proceeding. None of the participants reported an irritable bowel syndrome diagnosis on their health history questionnaire, and this information was also confirmed verbally. Eumenorrheic women were recruited using a questionnaire that verified (1) they had a cycle of $\geq$ 21 days and $\leq$ 35 days; (2) nine or more consecutive periods per year; (3) no menstrual irregularities; and (4) no hormonal contraceptive use in the 3 months prior to recruitment [12]. The oral contraceptive participants had been using a monophasic combined oral contraceptive pill for at least six months prior to study onset, and had not manipulated their cycle in the three months prior to the study. Details of the oral contraceptive brands are provided in Table 2.

Table 1
Participant characteristics for the three groups; men, women on combined monophasic oral contraceptive (OC) and eumenorrheic women (MC) (total n = 27). Data show mean ± SD.

Group (n)	Age (years)	Height (m)	Body Mass (kg)	BMI ($kg \cdot m^2$)	Cycle Length (day)	Ovulation (day)
Men (9)	30 ± 7	1.83 ± 0.07	87.9 ± 10.3	25.2 ± 2.9	–	–
MC (9)	28 ± 5	1.66 ± 0.05	62.8 ± 8.2	22.9 ± 3.1	29 ± 3	16 ± 2
OC (9)	25 ± 4	1.64 ± 0.07	66.6 ± 8.2	24.7 ± 2.1	28*	–

* 21 days pill and 7-day pill-free. MC = menstrual cycle group. OC = oral contraceptive group.

2.2. Study design & testing dates

All participants attended the laboratory for three experimental sessions. Men attended the laboratory-three times over six weeks with a two week washout between each trial. The MC group attended the laboratory during the early-follicular phase (Day 6 ± 1), ovulation [Day 14 ± 1, (2 ± 1 day before positive ovulation test)] and in the mid-luteal phase [Day 23 ± 3 (7 ± 1 day following positive ovulation test)]. The three-step method was used to confirm MC phases [28], with calendar tracking (2 months prior; Clue, https://helloclue.com/), ovulation tests (OneStep Ovulation Kits, China) and venous blood samples taken on testing dates for analysis of 17-β estradiol and progesterone [12,28]. Ovulation was confirmed if 17-β estradiol was higher in ovulation than mid-luteal and progesterone < 2.04 $ng \cdot mL^{-1}$ (~6 $nmol \cdot L^{-1}$). The mid-luteal phase was confirmed by progesterone above 5 $ng \cdot mL^{-1}$ (16 $nmol \cdot L^{-1}$) [16,28]. The OC group attended the laboratory-three times over a pill cycle. Once during the Pill-free phase (Day 6 ± 1), once during pill-1 phase (Day 14 ± 2), and once during the pill-2 phase (Day 21 ± 2) of the 28-day pill cycle. Participants verbally confirmed they had taken their pill every day throughout the cycle. Women participants completed the experiment in a maximum of two consecutive menstrual cycles. An online randomization tool (https://www.randomizer.org) was used to determine the menstrual cycle phase /pill phase in which participants began the experiment.

2.3. Pre-trial controls and measures

All participants were asked to complete an irritable bowel (GI-IBS) symptom questionnaire before starting the study [36]. Laboratory visits were preceded by a 12-h fast and participants were asked to refrain from caffeine and alcohol for 12 h before, and exercise for 24 h before attending the laboratory.

2.4. Experimental session protocol

Participants arrived at the laboratory in the morning (08:00 ± 00:30) and voided their bladder. Stature and body mass (Seca stadiometer, Seca scales, Germany) were measured and used to calculate body mass index (BMI). Participants then rested for 20 min on a bed in a semi-fowler position. Participants in the MC and OC groups were asked to complete a menstrual cycle symptom questionnaire adapted from the symptoms listed in Martin et al., [23]. All participants then consumed the L:R sugar drink (5 g Lactulose (Lactulose 3.3 g/5 mL Oral Solution, Boots, UK), 2 g L-Rhamnose [L-rhamnose Monohydrate, Apollo Scientific], 50 mL distilled water)), before a 20 mL venous blood sample was drawn. After the blood draw, participants left the laboratory and completed a 4-h fasted urine collection. During this time participants were asked to remain rested. Adherence was confirmed verbally. All urine was collected in a 2 L sterile urine container (Sarstedt, UK) and kept cold for the duration of the collection period.

2.5. Ex-vivo Protocol

Whole blood (15 mL) was syringed into sodium citrate containing tubes (Sarstedt, UK) which were left on rollers (Coulter mixer, UK) before stimulation with LPS (Lipopolysaccharide for E coli (055-B5; Sigma L4005- 100 mg) diluted in endotoxin free water (HyClone™ Water, Cell Culture Grade (Endotoxin-Free) Fisher Scientific). Whole blood from the sodium citrate tubes was pipetted into four 1.0 mL aliquots and 10 μL LPS (final concentration of 100 $\mu g \cdot mL^{-1}$) was added. Tubes were immediately submerged in a water bath (Fisher Scientific) for 6 h in either 37.0 °C (37.06 ± 0.05 °C) or 40.0 °C (40.01 ± 0.04 °C). Once the samples were removed from the water bath, they were centrifuged at 5,600 g for 5 min (Centurion scientific *C*2 series, CamLab, UK). Citrate plasma was then aliquoted and stored at −80 °C until analysis. Due to difficulty obtaining consistent blood samples from one

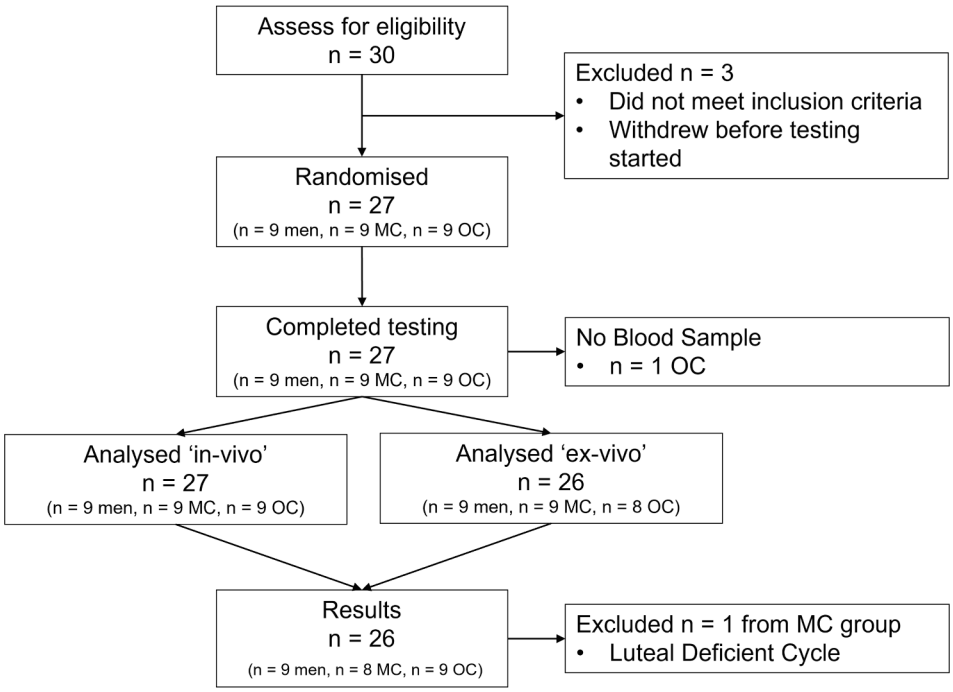

Fig. 1. CONSORT diagram showing recruitment and testing. Where MC = menstrual cycle group and OC = oral contraceptive group.

Table 2
Monophasic combined oral contraceptives taken by the OC group including progestin type and concentration and oestrogen type and concentration (total n = 9).

Brand	n	Progestin Type	Progestin Concentration	Oestrogen Type	Oestrogen Concentration
Microgynon, Rigevidon, Levest*	7	Levonorgestrel	0.15 mg	Ethinyl estradiol	30 μg
Loestrin 30	1	Norethisterone	1.50 mg	Ethinyl estradiol	30 μg
Marvelon**	1	Desogestrel	0.15 mg	Ethinyl estradiol	30 μg

* Microgynon, Rigevidon & Levest all contain the same active ingredients.
** excluded for ex-vivo analysis due to difficulties with the venous blood sample.

participant, venous blood samples for the OC group are presented as n = 8.

2.6. Serum hormone analysis

The remaining 5 mL of venous blood was syringed into a 5 mL tube containing a clot activator; this was left for 30 min to clot before being centrifuged at 1,500 g for 15 min (Heraeus Biofuge Primo Centrifuge, Thermo Scientific, UK). The serum was aliquoted and stored at −80 °C. Concentrations of 17-β estradiol & progesterone were determined via ELISA (IBL-international 17-β estradiol and progesterone). The intra-plate coefficient of variation (CV) across both plates was 6.6 %.

2.7. Quantification of urinary lactulose and rhamnose

Urine was weighed to determine total urine production over the 4-h collection period, then mixed thoroughly before four 1.5 mL aliquots were taken and stored at −80 °C. Lactulose was quantified using an Enzyme Immunoassay (EIA) (K-LACTUL; Megazyme, Wicklow, Ireland) with some deviations from the manufacturer's instructions. The supplied glucose/fructose (0.2 mg/ml) standard was serially diluted 1:2. Fifty-five microliters of blank, standard, or urine were added to 96-well microtiter plates, followed by 55 μL of Tris-acetate-EDTA (TAE) buffer (pH 7.6), 10 μL of β-galactosidase, 10 μL imidazole buffer, and 10 μL –NAD+/ATP solution. The plate was mixed and incubated for 3 min at 37.0 °C, then read at 340 nm. The plate was next incubated at 37 °C for 2 h to allow galactosidase conversion of lactulose into free glucose and fructose. After incubation hexokinase, +G-6-P dehydrogenase solution was diluted 1:5 in TAE buffer, and 10 μL was added to all occupied wells. The plate was mixed and incubated for 5 min at 37 °C, then read at 340 nm. PGI was diluted at 1:5 in 0.5 TAE buffer, and 10 μL was added to the plate, mixed, and incubated at 37 °C (5 min) before a final reading at 340 nm. Rhamnose was quantified using a colorimetric assay according to the manufacturer's instructions (K-RHAM, Megazyme, Wicklow, Ireland). The concentration of lactulose and rhamnose detected in the urine sample (g/L) was multiplied by the volume of the 4-h collection (L) and expressed in grams (g). These values were used to calculate the percentage of the given dose (g) that was recovered, which was expressed as a ratio (Lactulose: Rhamnose).

2.8. Quantification of TNFα

Following *ex-vivo* stimulation, whole blood samples were analysed for TNFα via enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Duoset DY210, R&D Systems, Minneapolis MN). Samples were diluted 1:40 in 0.1 % bovine serum albumin and phosphate buffer saline. The optimal sample dilution was determined from prior in-house linearity and spike-recovery assessments. The intra-plate CV was 4.0 % across all plates and the inter-plate CV was 13.6 %.

2.9. Statistical analysis

All data were analysed with JASP (JASP (Version 0.14.1)). Data are

presented as mean (95 % confidence interval (CI)). All data were checked for normality before any statistical analysis was completed. L:R ratio data were analysed using a repeated-measures ANOVA (3 × 3) with the between-subject factor of the group (men vs MC vs OC) and a within-subject factor of the trial (Men; Trial 1 vs Trial 2 vs Trial 3, MC; early-follicular vs ovulation vs mid-luteal, OC; pill-free, vs pill-1 vs pill-2). The main effects were assessed with a Holm correction to control for multiple comparisons. TNFα was analysed using a repeated-measures ANOVA (3 × 3 × 2) with the between-subject factor of group (men vs MC vs OC) and a within-subject factor of the trial (Men; Trial 1 vs Trial 2 vs Trial 3, MC; early-follicular vs ovulation vs mid-luteal, OC; pill-free, vs pill-1 vs pill-2) and temperature (37.0 °C vs 40.0 °C). Summed IBS questionnaire data were analysed using a non-parametric Kruskal-Wallis test, summed MC symptoms were analysed using non-parametric Friedman's test with Conover post hoc comparisons and are expressed as median values. 17-β estradiol and progesterone concentrations were assessed for the MC group using a one-way repeated measures ANOVA with within-subject factor trial (early-follicular vs ovulation vs mid-luteal). To provide the reader with an objective indication of the magnitude of the differences, effect sizes were calculated as Cohen's *d* for multiple comparisons or as partial eta squared (η_p^2) for RM-ANOVA. For reference, values of 0.2, 0.5, and 0.8 correspond to small, medium, and large effect sizes for *d*, respectively and values of 0.01, 0.09, and 0.25 are considered to be small, medium, and large effect sizes for η_p^2 [10].

3. Results

Fig. 2 shows the oestrogen and progesterone concentrations for the MC groups (n = 9). Low progesterone (<5 ng·mL^{-1}) indicated one participant was luteal deficient so her data were excluded from statistical analysis. Progesterone concentrations for the remaining 8 participants were above the 5 ng·mL^{-1} concentration required to confirm the mid-luteal phase (13.0 ng·mL^{-1}, 9.3 to16.7 ng·mL^{-1}, mean, 95 % confidence interval). Progesterone concentration differed across the phases of the menstrual cycle ($F_{(2,14)} = 35.044$, $p < 0.001$, $\eta_p^2 = 0.834$), and was higher during the mid-luteal than early-follicular phases (by + 12.5 ng·mL^{-1},+8.4 to + 16.6 ng·mL^{-1}), $p < 0.001$, $d = 2.43$) and higher at mid-luteal than ovulation (by + 11.3 ng·mL^{-1},+7.3 to + 15.4 ng·mL^{-1}), $p < 0.001$, $d = 2.68$). Oestrogen concentrations differed across the phases of the menstrual cycle ($F_{(2,14)} = 7.767$, $p = 0.005$, $\eta_p^2 = 0.53$). Oestrogen was higher at ovulation than early-follicular (by + 42.0 pg·mL^{-1}, +24.3 to + 59.7 pg·mL^{-1}, $p = 0.061$, $d = 0.85$) and higher at mid-luteal than early-follicular (by + 66.2 pg·mL^{-1},+31.1 to + 101.4 pg·mL^{-1}, $p = 0.005$, $d = 1.38$).

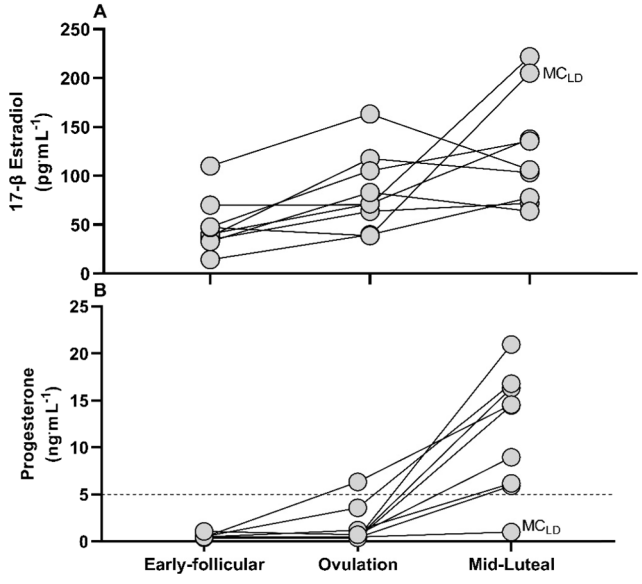

Fig. 2. A) 17-β estradiol concentrations in MC participants (n = 9) across the three phases of the menstrual cycle B) concentrations in MC participants (n = 9) across the three phases of the menstrual cycle. MC_{LD} = luteal deficient participant.

3.1. In vivo gastrointestinal permeability

The lactulose rhamnose (L:R) ratio was different between the three groups (group effect; $F_{(2.23)} = 6.187$, $p = 0.007$, $\eta_p^2 = 0.35$, Fig. 3). The OC group had a higher L:R ratio compared to men (by + 0.005, +0.001 to + 0.008,$p = 0.007$, $d = 0.68$) and MC (by + 0.003, +0.001 to + 0.007, $p = 0.061$, $d = 0.45$). No difference was observed between MC and men ($p = 0.311$, $d = 0.20$). Results were similar across trials (trial main effect, $F_{(2,46)} = 0.483$, $p = 0.62$, $\eta_p^2 = 0.02$), and there was no trial × group interaction ($F_{(4,46)} = 1.626$, $p = 0.18$, $\eta_p^2 = 0.12$).

As compared to Men (median summed score 14 (range 13–33)), median summed IBS symptoms were higher in OC (median summed score 18 (range 13 – 44)) and MC (median summed score 26 (range 13 – 32); Fig. 4). However, no overall difference was found between the groups ($\chi^2_{(2)} = 3.890$, $p = 0.143$). Menstrual cycle symptoms were different across the menstrual cycle ($\chi^2_{(2)} = 8.706$, $p = 0.013$). Conover post hoc tests show that greater symptoms were observed in the early-follicular phase (median score 38) compared to ovulation (median score 22, $p = 0.027$) and mid-luteal (median score 22, $p = 0.017$). Menstrual cycle symptoms were also different across pill cycle ($\chi^2_{(2)} = 7.032$, $p = 0.030$). Conover post hoc test shows that greater symptoms were observed in the pill-free phase (median score 34) than pill-1 (median score 20, $p = 0.027$), and pill-2 (median score 22, $p = 0.044$).

3.2. Whole blood LPS treatment

Baseline concentrations of TNFα (e.g. unstimulated) were below the 15.6 pg·mL^{-1} detection limit of the assay for all participants. Following treatment, plasma TNFα concentrations were different between the three groups (group main effect $F_{(2,22)} = 9.137$, $p < 0.001$, $\eta_p^2 = 0.45$, Fig. 5). Holm post hoc tests show that TNFα concentration was higher in men than OC (by + 66 %, +29 to + 104 %), $p = 0.004$, $d = 0.70$) and MC (by + 71 %, +12 to + 130 %, $p = 0.003$, $d = 0.76$). No differences were seen between trials (trial main effect, $F_{(2,44)} = 2.241$, $p = 0.118$, $\eta_p^2 = 0.09$), and there was no group × trial interaction ($F_{(4,44)} = 1.415$, $p = 0.245$, $\eta_p^2 = 0.11$). There was a main effect for temperature ($F_{(1,22)} = 28.725$, $p < 0.001$, $\eta_p^2 = 0.57$), with post hoc analysis showing that simulated hyperthermia led to a + 23 % (+14 to + 33 %) increase in TNFα compared to the thermally neutral condition. There was a trial × temperature interaction ($F_{(2,44)} = 4.012$, $p = 0.25$, $\eta_p^2 = 0.15$), with Holm post hoc tests showing TNFα concentrations were higher in all groups following the hyperthermic condition ($p < 0.001$). There was no group × temperature interaction ($F_{(2,22)} = 0.288$, $p = 0.752$, $\eta_p^2 = 0.02$) and no interaction between group × trial ($F_{(4,44)} = 1.415$, $p = 0.245$, $\eta_p^2 = 0.11$).

4. Discussion

The present study assessed *in-vivo* GI permeability and *ex-vivo* TNFα responses to monocyte treatment with LPS and hyperthermia in men, eumenorrheic women (MC), and in women taking oral contraceptives (OC). We present four main findings. First, *in-vivo* data indicate that resting GI permeability, assessed using a dual sugar absorption technique (ratio of lactulose to rhamnose excretion) was higher in oral contraceptive users compared to MC women and men. Second, as compared to MC and OC women, men had less variability in their ratio of lactulose and rhamnose excretion. Third, *ex-vivo* monocyte stimulation with LPS induced greater TNFα release in men as compared to MC and OC women, regardless of the incubation temperature. Fourth, it appears oral contraceptive use may moderate monocyte response to *ex-*

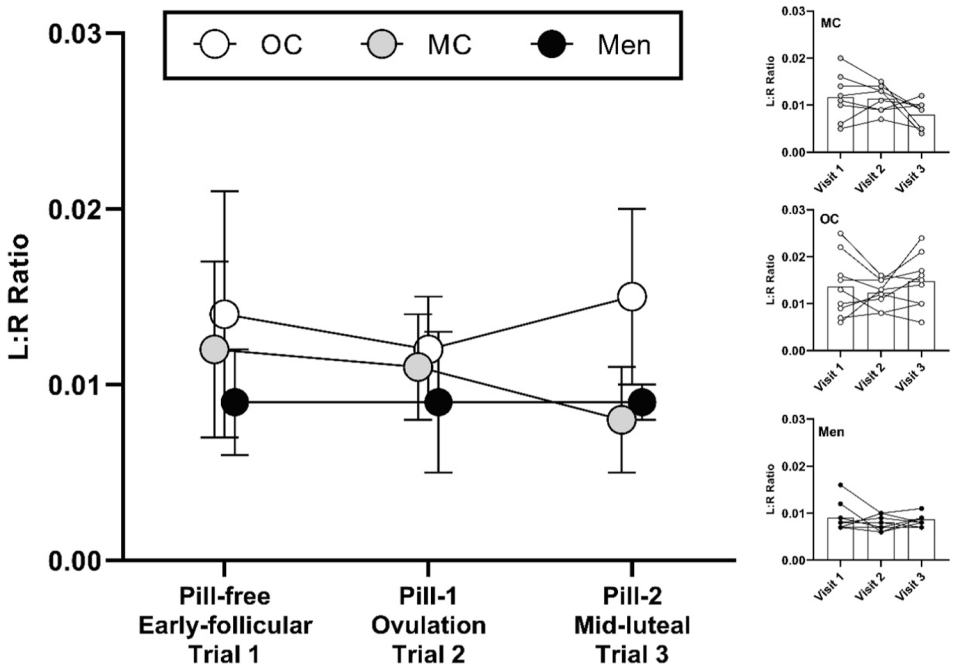

Fig. 3. L:R ratio mean ± SD presented for the three groups (MC (n = 8), OC (n = 9) and Men (n = 9)) across the three visits. The inset graphs show individual data and visit mean for MC (grey dots), OC (white dots) and Men (black dots).

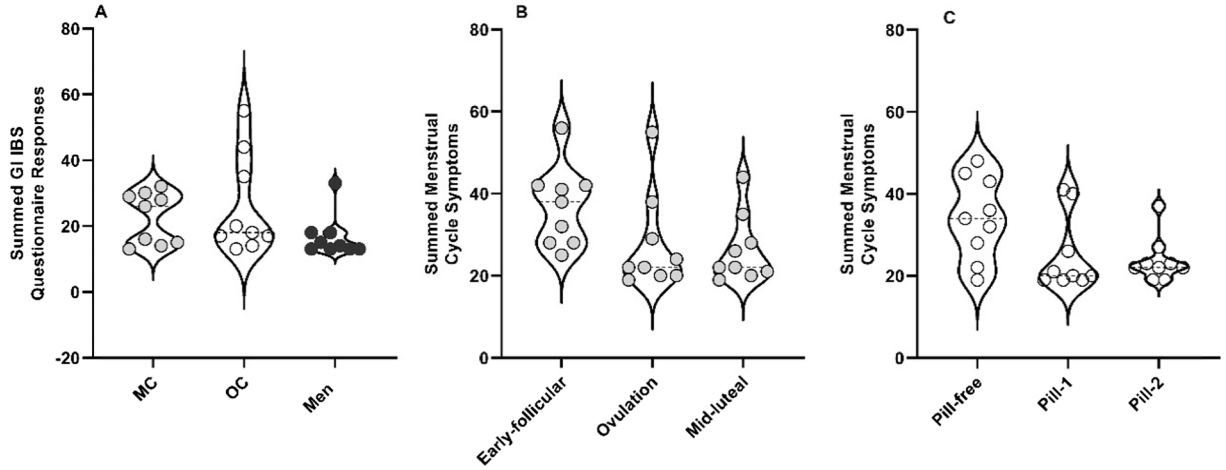

Fig. 4. Violin plots showing individual symptom scores, the dotted line indicates the median score and the violin tails demonstrate the distribution of scores. A) GI-IBS symptoms across the three groups Men (black dots), MC (grey dots), OC (white dots), (total n = 27), B) Menstrual cycle symptoms across the three menstrual cycle phases in the MC group (n = 9) and C) menstrual cycle symptoms across the pill cycle in the OC group (n = 9).

vivo LPS challenge, as TNFα release was consistent across time points in the OC group. In contrast, TNFα release was more variable in the MC group.

4.1. In-vivo differences in gastrointestinal barrier permeability

To summarise the *in-vivo* experiment, our data show that when rigorous experimental controls (e.g. overnight fast, physical activity controls, same time of day) are used, the lactulose rhamnose dual sugar absorption test displays low variability across 3 repeated experimental visits when assessed in healthy young men (co-efficient of variation [CV] across the 3 visits ~ 14 %, range 12–18 %), and is similar to recently reported reliability data (CV ~ 12 %, Ogden et al., 2020). However oral contraceptive users displayed greater variation in GI permeability when tested at the different phases of the pill cycle (CV ~ 24 %, range 17 – 32 %). The largest variation observed between the pill-free and pill-2 trials (CV ~ 32 %), and least variation observed between pill-1 and pill-2 (CV ~ 17 %). Eumenorrheic women displayed the greatest variability in responses when tested across the different phases of the menstrual cycle (CV ~ 28 %, range 16 – 35 %). The least variation in response was observed between the early follicular and ovulation phase (CV ~ 16 %), and the greatest variation was observed between mid-luteal and follicular phases (CV ~ 35 %).

Women who had been using oral contraceptives for > 6 months presented with higher L:R ratios across all phases of the pill cycle. No difference in the L:R ratio was observed between the pill-free to pill phases, which could be due to chronic downregulation of endogenous hormones. This is supported by a prior report (Elliott-Sale et al. 2020) that showed lower oestrogen concentrations during the pill-free phase (~38 pg·mL^{-1}) and pill phase (~16 pg·mL^{-1}), as compared to eumenorrheic women in the follicular phase (48 pg·mL^{-1}, 28–69 pg·mL^{-1}). Although we are the first to assess the L:R ratio across the oral

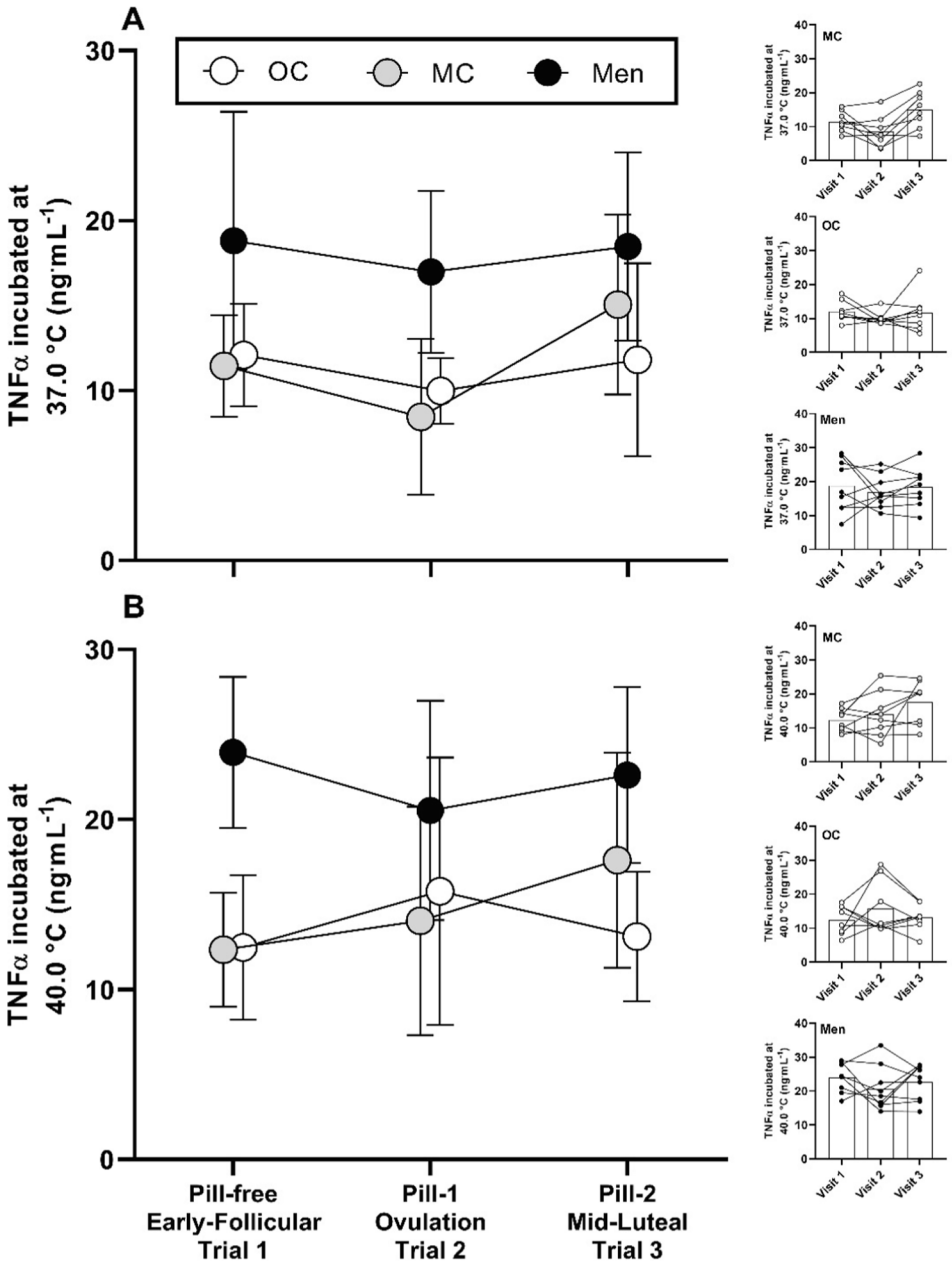

Fig. 5. *Ex-vivo* TNFα mean ± SD responses across the three groups and three phases trials (MC, n = 8; OC, n = 8, Men, n = 9). A) Incubated at body temperature (37.0 °C) B) incubated in hyperthermia (40.0 °C). The inset graphs show individual data and visit mean for MC (grey dots), OC (white dots) and Men (black dots).

contraceptive phases, evidence from multiple *meta*-analyses suggest there is an association between Crohn's disease and chronic oral contraceptive use [11,20]. In fact, it appears that chronic oral contraceptive use increases the relative risk of Crohn's disease by approximately 50 % [11,20]. These studies suggest that modifications in colon function, in conjunction with elevations in thrombosis risk, may contribute to multifocal gastrointestinal infarction and progression in disease pathology. Subjective menstrual cycle symptoms in the present study were highest during the pill-free week, which agrees with prior work [19]. While it has been suggested that the transition from synthetic hormones (in the pill phases) to the pill free week may impact tight junction integrity, this was not observed in the present study, where no change in the L:R ratio was detected between the Pill free, Pill 1 and Pill 2 phases in OC.

Previously, no differences in GI permeability had been observed between the early-follicular and mid-luteal phase of the menstrual cycle in healthy pre-menopausal women Torella et al., (2007 Lambert et al., [22]. However, the menstrual cycle phase was not characterised in either study (by hormones or by ovulation testing), so those results cannot be directly attributed to a specific hormonal profile or phase [12]. In the present study, we took great care in profiling each participant's menstrual cycle, to ensure all data were collected in the optimal period for clinical assessment of GI barrier permeability. For example, the early-follicular phase was tested on cycle day 6 ± 1 and confirmed via low hormone concentrations for oestrogen and progesterone. The mid-luteal phase was confirmed via a progesterone cut-off of 5 $ng{\cdot}mL^{-1}$ (16 $nmol{\cdot}L^{-1}$), and 1 participant was identified as luteal deficient and excluded from data analysis. Despite stringent criteria and individual participant tracking, accurately determining ovulation was more challenging, with only 4/8 participants meeting the pre-set criteria (oestrogen higher than during the mid-luteal phase and progesterone lower than 2.04 $ng{\cdot}mL^{-1}$, [17,32], although all participants did present with a positive ovulation test. The use of more sensitive ovulation tests may improve the ability to detect this phase accurately and reliably.

Eumenorrheic women in the current study displayed the greatest variability in responses when tested across the different phases of the menstrual cycle (CV ~ 28 %, range 16 – 35 %). The increased GI barrier permeability occurred in the early-follicular phase alongside high GI and MC symptoms and low oestrogen and progesterone however, the change in GI barrier permeability did not meet conventional levels of statistical significance. It should also be noted that the L:R change that was shown in the present study was small (L:R change from early-follicular to mid-luteal 0.004), and the clinical significance of this difference is likely small and of little physiological consequence. However, there is no consensus within the literature regarding the minimal clinically important difference for dual sugar absorption testing. Despite the lack of statistically significant findings, the above information regarding the expected variation in baseline L:R ratio when tested between sexes, across pill phases or menstrual cycle phases provides valuable information for clinicians and researchers employing this diagnostic test. For example, if basal L:R ratio is being assessed at multiple time points across a study or following an intervention, oral contraceptive users should be tested during the pill 1/pill 2 phase to minimize the potential variation caused by when transitioning back onto synthetic hormones following the pill free phase. Similarly, to minimise variation in baseline L:R ratio in Eumenorrheic women, and where it is not experimentally feasible to test during the same phase on the MC, repeat visits during the mid-luteal and ovulation phases may reduce the potential variation. In contrast, the variation in men was largely consistent between all experimental visits.

Interestingly, menstrual cycle symptoms were higher in the early-follicular phase (as compared to ovulation or the mid-luteal phase), meaning those subjective data were shown to agree with objective measurements of greater GI barrier permeability. Thus, the marginally elevated L:R ratio and increased prevalence of menstrual symptoms could be related to reductions in 17β- estradiol concentrations (early-follicular oestrogen 48 $pg{\cdot}mL^{-1}$ vs mid-luteal oestrogen 115 $pg{\cdot}mL^{-1}$). Taken together, the data from oral contraceptive users and eumenorrheic women may suggest that chronic pill use (>6 months) influences GI barrier permeability, which could be due to suppression of oestrogen due to the chronic use of the oral contraceptive pills and warrants further study aimed specifically at addressing this observation.

4.2. Ex-vivo monocyte response to LPS challenge

To examine the effects of sex, menstrual cycle phase, and oral contraceptive use on monocyte function (TNFα release), we conducted an *ex-vivo* experiment in which whole blood was stimulated with LPS with and without additional hyperthermia at each phase of the menstrual cycle/oral contraceptive cycle. We show that men released more TNFα in response to the LPS challenge, regardless of temperature, a finding in keeping with most [1,5,18,30], but not all [34] previous studies. Oestrogen contributes to the sex differences of immune responses (Bhatia et al., 2014), and the heightened TNFα release in men has been suggested to be a result of a lower number of oestrogen receptors in monocytes [25,30]. Some of the sex-dependant responses to LPS stimulation are also likely explained by the fact that LPS binds to TLR-4, which are more heavily expressed in monocytes and neutrophils from males, as compared to females [25]. This could explain why men are in comparison more biased towards pro-inflammatory responses and why men have worse prognoses following sepsis, severe trauma or COVID-19 [21,35].

With regards to menstrual cycle effects, the TNFα response was similar between both the OC and MC groups, with little variation observed across the pill cycle. However, TNFα release more variable across the MC and was greater in the mid-luteal phase of the menstrual cycle in comparison to both the early-follicular (+37 %) and ovulation phase (+45 %), despite not meeting conventional levels of statistical significance. It is possible that monocytes are more sensitive to endotoxin stimulation during the mid-luteal phase when high concentrations of oestrogen and progesterone are present [4]. At rest without stimulation, increases in cytokines (IL-1 β, Il-1, TNFα) and LPS inducing IL-1β producing monocytes have been measured as higher in the mid-luteal compared to the early-follicular phase in plasma samples [6,9,27]. The percentage of TNFα and IL-1β producing cells were increased during the mid-luteal phase (6.1 days post-LH surge) when compared to the early-follicular (day 6.9) phase when monocytes were stimulated with 2.0 $\mu g{\cdot}mL^{-1}$ LPS for 4 h [4]. However, this has not been consistently shown, with Schwarz et al., [29] and Temple et al. [30] finding either no response or lower concentrations of pro-inflammatory cytokines during the mid-luteal phase. The contrasting results may have been due to the lack of menstrual cycle characterisation procedures.

To summarise the two arms of the study, we demonstrate that the L:R ratio has greater variation in both eumenorrheic women and women taking an oral contraceptive relative to healthy men. The greatest variation within the menstrual cycle coincided with low concentrations of oestrogen and progesterone, and increased subjective reporting of MC symptoms. OC women had higher permeability ratios than men or MC however this was unaffected by the pill phases. When whole blood was stimulated with LPS men had a heightened TNFα response than MC or OC, potentially due to lower numbers of oestrogen receptors and TLR-4 expression in monocytes. TNFα responses were more variable across the MC in eumenorrheic women however this didn't reach conventional levels of significance. In women who take an oral contraceptive, TNFα responses were unaffected by the change from pill-free to pill phases. When cells were incubated in hyperthermia (40.0 °C) TNFα increased across all groups. However, this did not impact further upon responses across the MC or OC cycle.

5. Conclusion

Over 3 repeated laboratory experiments, men displayed a lower variation in the excretion of lactulose and rhamnose, which suggests that GI barrier permeability is relatively stable under resting conditions. In contrast, oral contraceptive users had the highest permeability ratios which were unaffected by the change from pill-free to pill phases. In the MC group permeability ratios were more variable with the highest L:R ratios in the early-follicular phase alongside low oestrogen and progesterone and high MC symptoms scores. Larger TNFα responses were seen in men than OC or MC, TNFα was unaffected by the change from pill-free to pill phase in OC but were more variable across the MC. These results suggest that both intestinal permeability and inflammatory responses are affected by both sex, menstrual cycle and oral contraceptive use, highlighting the need for more *in-vivo* research in these populations.

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Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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