Investigating the Role of Lymphocytes and Fatty Acid Amide Hydrolase in Term Human Pregnancy

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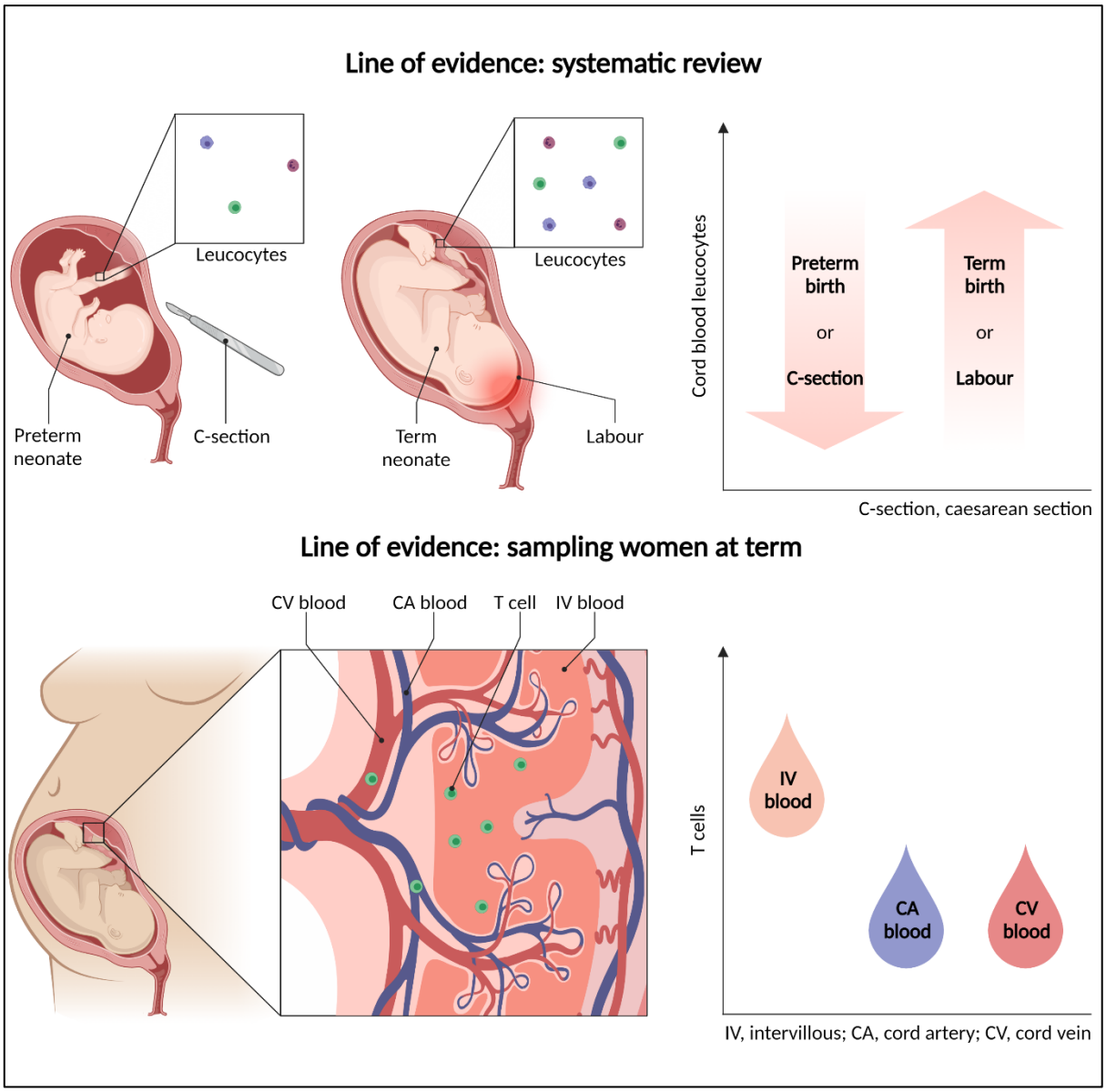
Graphical abstract



Acknowledgements

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Introduction

Rationale for research

Preterm labour (PTL) is defined as labour before 37 weeks and is a leading cause of maternal and neonatal morbidity and mortality, with a worldwide incidence of about 10.6% of births per year (1). Evidence suggests labour is an inflammatory process and that spontaneous PTL may result from premature activation of the same inflammatory pathways activated in labour at term (2, 3). Hence, increased insight into the events preceding human labour is necessary to improve prediction and prevention of PTL.

Control of parturition

Our poor grasp of the mechanisms of parturition stems from practical and ethical difficulties associated with studying human parturition *in vivo* (4). Consequently, our understanding of labour comes mostly from rodent, sheep and primate models (Fig. 1). As these may not accurately reflect labour in humans (4, 5) it is imperative that hypotheses generated from animal experimentation be tested in humans (6).

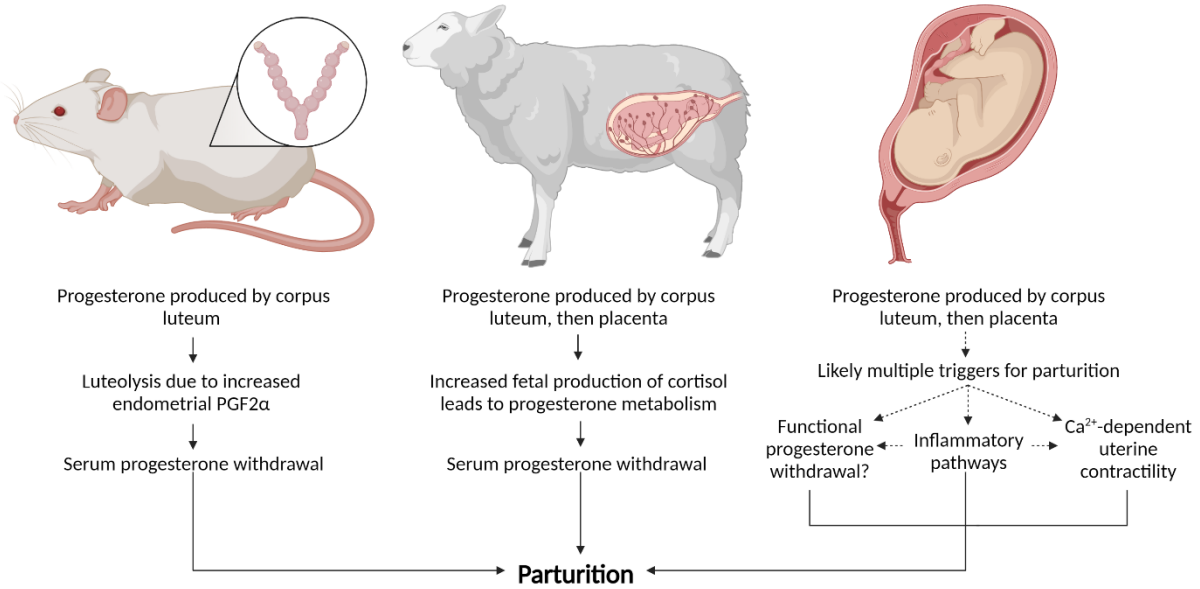

Figure 1: A comparison of the mechanisms of parturition in sheep, rodents and humans. Murine parturition results from the luteolytic effect of endometrial PGF2α and a corresponding decline in serum progesterone. Like humans, sheep experience a "luteal-placental shift" of progesterone production during pregnancy, but parturition occurs due to activation of the fetal hypothalamic-pituitary-adrenal axis and increased placental metabolism of progesterone. In humans, serum progesterone levels are unaffected by the onset of labour and there are likely multiple triggers for parturition, including functional progesterone withdrawal, inflammation and electrophysiological changes in myometrial cells. PGF2α, prostaglandin F2α. Created with BioRender.com.

The placenta has numerous functions essential for gestational success, and is likely involved in initiating labour (7). Blood flows between the placenta and fetus via the umbilical cord, typically containing one cord vein (CV) and two cord arteries (CA), while maternal blood enters through the endometrial arteries and pools in the intervillous (IV) spaces (8) (Fig. 2). Sampling CA blood is more challenging than CV due to the smaller lumen, thicker wall and reduced volume of blood available for sampling following delayed cord clamping (9). Consequently, most previous studies of the fetal circulation relate to CV only (10, 11) meaning differences in blood derived from the fetus (CA) and blood from the placenta (CV) cannot be identified. Separate measurements of the CA, CV and maternal circulation (IV) could help to localise changes that occur at the time of labour and birth (7).

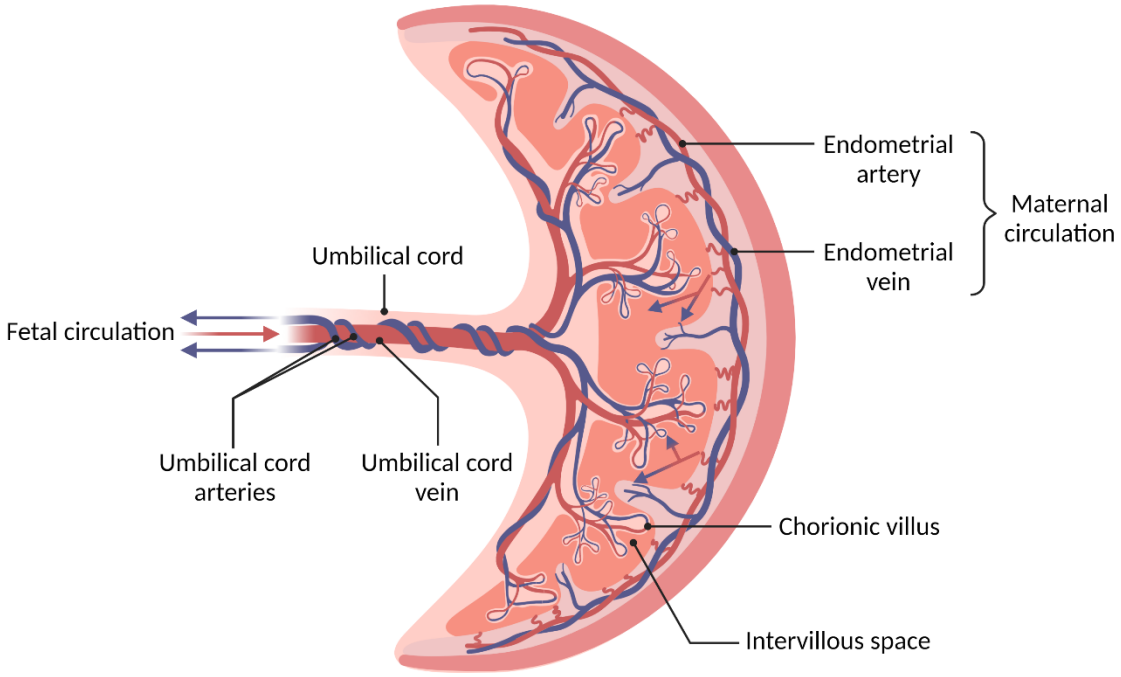

Figure 2: Schematic representation of blood flow through the human placenta showing the separate fetal and maternal circulations. Oxygen-rich (red) maternal blood flows into the placenta via the endometrial arteries, pools in the intervillous space flows out of the placenta via the endometrial veins. The fetal surface of the placenta is supplied by the umbilical arteries (usually two) which bring deoxygenated (blue) blood from the fetus to the chorionic villi, allowing gas exchange. Oxygenated blood returns to the fetus via a single umbilical vein. Created with BioRender.com.

The endocannabinoid system

Endocannabinoids (eCBs) are non-classical neurotransmitters, of which anandamide (AEA) is the best characterised (12). Maternal plasma AEA levels decline throughout pregnancy before rapidly increasing during active labour, with AEA levels approximately four times higher during labour compared to non-labouring term levels (13). A study investigating levels

of AEA at term found higher concentrations in placenta versus fetal membranes, CV versus CA and maternal plasma versus CA (14), while a metabolomic study by Birchenall *et al.* found significantly increased AEA in the cord plasma of women who laboured compared to those who underwent caesarean (c-section). Together, these data suggest local production or transportation across the placenta (10).

Human peripheral lymphocytes may play a major role in AEA degradation as they possess CB-receptors and express the catabolic enzyme fatty acid amide hydrolase (FAAH) (15). However, no studies have investigated how lymphocytic FAAH activity is affected by labour at term in humans. Further research is needed to distinguish fetal (CA), placental (CV) and maternal (IV) circulations to localise the trigger for human labour.

Hypothesis

The trigger for labour in humans involves changing activity of lymphocytic FAAH in the uterus, coordinating an increase in eCB levels in cord blood which in turn stimulates an inflammatory response in the mother, resulting in myometrial contractions.

Aims and objectives

Aim	Objective
1. To review the literature to determine whether there is an association between cord blood lymphocytes and gestational age (GA), type of labour onset or mode of delivery (MOD).	1. To achieve Aim 1, a systematic review was conducted to identify relevant studies, followed by synthesis, quality analysis and meta-analysis.
2. To compare T lymphocyte populations and FAAH levels in IV (maternal), CA (fetal) and CV (placental) blood in order to characterise maternal-fetal interactions and the involvement of the endocannabinoid system at term.	2. To achieve Aim 2, samples were taken from women undergoing c-section at term and used for flow cytometry and FAAH quantification.

Table 1: Aims and objectives of the study.

Systematic review and meta-analysis of current literature

Materials and methods

Through a systematic review and meta-analysis, we aimed to evaluate the literature to investigate the association between cord blood lymphocytes and (i) GA, including preterm birth (PTB); (ii) type of labour onset and (iii) MOD.

The following electronic databases were searched in October 2020: MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials, CINAHL and ClinicalTrials.gov. Search terms were chosen according to the PICO or PECO framework (Population, Intervention/Exposure, Comparator and Outcome) (16) (Table 2).

P	Neonates (of any gestation)	((prematur* ***or*** postmatur*) ADJ3 (neonat* ***or*** neo-nat* ***or*** newborn ***or*** infant* ***or*** baby ***or*** babies ***or*** labor ***or*** labour)).ti,ab
E	GA at delivery	(Preterm ***or*** pre-term ***or*** postterm ***or*** Post-term).ti,ab ***or*** ("gestational age" ADJ3 (delivery ***or*** birth)).ti,ab ***or*** ((small ***or*** large) ADJ1 "gestational age").ti,ab ***not*** (review).pt ***or*** ("meta analysis").pt ***or*** (news).pt ***or*** (comment).pt ***or*** (editorial).pt ***or*** ("cochrane database of systematic reviews").so ***or*** ("systematic review" ***or*** "literature review").ti
	Type of labour onset	((trial ***or*** induc*) ADJ1 labor).ti,ab
	MOD	((mode ***or*** method ***or*** route) ADJ2 (delivery ***or*** childbirth ***or*** birth)).ti,ab ***or*** (cesarea* ***or*** caesarea*).ti,ab ***or*** ("c section*").ti,ab ***or*** (postcesarea* ***or*** postcaesarea*).ti,ab ***or*** ((vaginal ***or*** normal) ADJ3 (birth ***or*** delivery)).ti,ab ***or*** (forceps).ti,ab ***or*** (ventouse).ti,ab ***or*** ("suction cup").ti,ab ***or*** (kiwi ***or*** malmstrom).ti,ab ***or*** (vacuum).ti,ab ***or*** (odon).ti,ab ***or*** ((operative ***or*** instrumental ***or*** assisted) OADJ1 (delivery ***or*** birth)).ti,ab
C	*Not applicable*	
O	Lymphocytes	(Lymphocyte*).ti,ab ***or*** ("t cell" ***or*** "t cells" ***or*** tregs).ti,ab ***or*** ("b cell" ***or*** "b cells").ti,ab ***or*** ("white cell" ***or*** "white cells").ti,ab ***or*** (Neutrophil*).ti,ab ***or*** (Macrophage*).ti,ab ***or*** ("natural killer cell*").ti,ab ***or*** (CD4).ti,ab ***or*** (CD8).ti,ab

Table 2: Search terms and medical subject heading (MeSH) terms used to search MEDLINE, EMBASE, CENTRAL, CINAHL (via EBSCO) and ClinicalTrials.gov. MeSH terms and key words were chosen according to the PECO framework (Population, Exposure, Comparator and Outcome). Restrictions included animal studies, reviews, secondary research and comment or opinion pieces.

Studies were assessed against a set of *a priori* inclusion and exclusion criteria (Table 3).

Inclusion criteria	Exclusion criteria
Randomised controlled trial, prospective or retrospective cohort, case-control or cross-sectional studies	Studies not published in English
Studies reporting lymphocyte numbers (total and/or subpopulations) in cord blood, as measured in the immediate post-partum period	Studies not conducted in humans
Studies providing information on GA at delivery (including PTB), type of labour onset or MOD	Studies not related to labour, PTB or GA
	Studies on subjects with maternal/neonatal conditions (e.g. infection, pre-eclampsia or diabetes), which do not also include separable data on healthy subjects

Table 3: Inclusion and exclusion criteria for the systematic review. PTB, preterm birth; GA, gestational age; MOD, mode of delivery.

Titles and abstracts were screened for relevance by two reviewers in a blinded fashion. Articles identified as potentially relevant underwent full-text review. Two reviewers independently extracted the data using a bespoke data extraction form. Raw counts and percentages of lymphocytes were extracted from tables and text.

Statistical analysis was performed using Review Manager 5. Data were pooled through a random-effects meta-analysis and presented as mean differences (MD) or standardised mean differences (SMD) with associated 95% Confidence Intervals (CI). Heterogeneity was assessed using the I^2 statistic and the quality of meta-evidence for significant comparisons rated using the GRADE approach as very low, low, moderate or high. A *P* value $\leq$0.05 was considered statistically significant.

Results

The search returned 5,836 articles, after automated removal of duplicates; overall, 9 articles were suitable for meta-analysis (Fig. 3). All studies were non-randomised. The primary exposure was GA at delivery in four studies and MOD in four studies; one study considered both GA and MOD as exposure variables. No studies assessed type of labour onset. As leucocyte and total lymphocyte counts were the most widely reported data fields, these were the outcome variables.

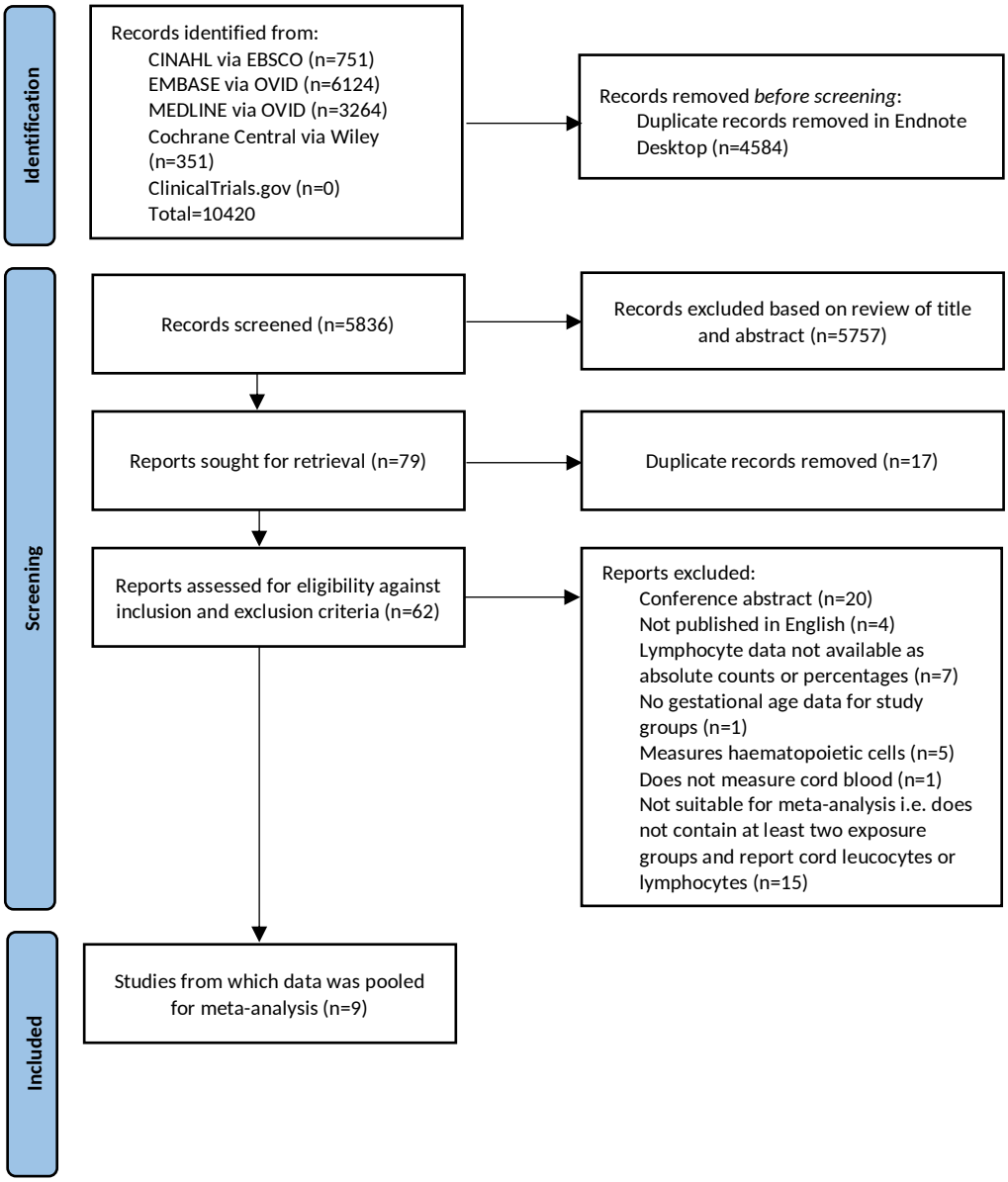

Figure 3: Screening and selection of studies included in the systematic review and meta-analysis, adapted from PRISMA Flow Diagram (85).

Compared with preterm, term babies had higher numbers of cord leucocytes (SMD 0.67, 95% CI 0.37–0.97, 4 studies, 2804 babies, *P*<0.0001) and tended to have higher cord lymphocytes, although this was not statistically significant (SMD 0.33, 95% CI -0.15–0.80, 4 studies, 2941 babies, *P*=0.18) (Fig. 4). Moderate heterogeneity existed across studies in the leucocyte analysis (I^2=44%). Heterogeneity was high for studies in the lymphocyte analysis (I^2=86%).

Meta-analysis of studies concerning MOD revealed newborns delivered after labour (versus c-section) had higher numbers of cord leucocytes (SMD 0.95, 95% CI 0.18–1.73, 5 studies, 443 babies, *P*=0.02). Levels of cord lymphocytes were not significantly different between the two groups (SMD -0.35, 95% CI -2.09–1.38, 5 studies, 443 babies, *P*=0.69) (Fig. 5).

Heterogeneity was high for both leucocyte and lymphocyte analyses (I^2=89% and 98%, respectively).

The GRADE approach was used to assess the quality of meta-evidence for comparisons with *P*<0.05. Evidence quality was low for the effect of GA and very low for MOD, due to substantial heterogeneity (inconsistency) and the wide CI (imprecision). This indicates the true effect may be markedly different from the estimated effect (17).

No. of studies	Quality of studies (max. 9)	SMD [95% CI]	Indirectness	Inconsistency	Imprecision	Other	Quality of evidence
Cord leucocytes (term versus preterm newborns)							
4	6–8	0.56 [0.30, 0.82]	None	Low	None	None*	⊕⊕ Low†
Cord leucocytes (newborns delivered by labour versus c-section)							
5	3–7	0.95 [0.18, 1.73]	None	High	High	None*	⊕ Very low due to inconsistency, imprecision†

Table 4: GRADE quality rating of meta-evidence for significant findings. *Publication bias was considered unlikely due to the comprehensive search strategy and as no of conflicts of interest were declared by the study authors. †GRADE assigns an *a priori* ranking of "low" to observational studies (all studies included in this review). SMD, standardised mean difference; CI, confidence interval.

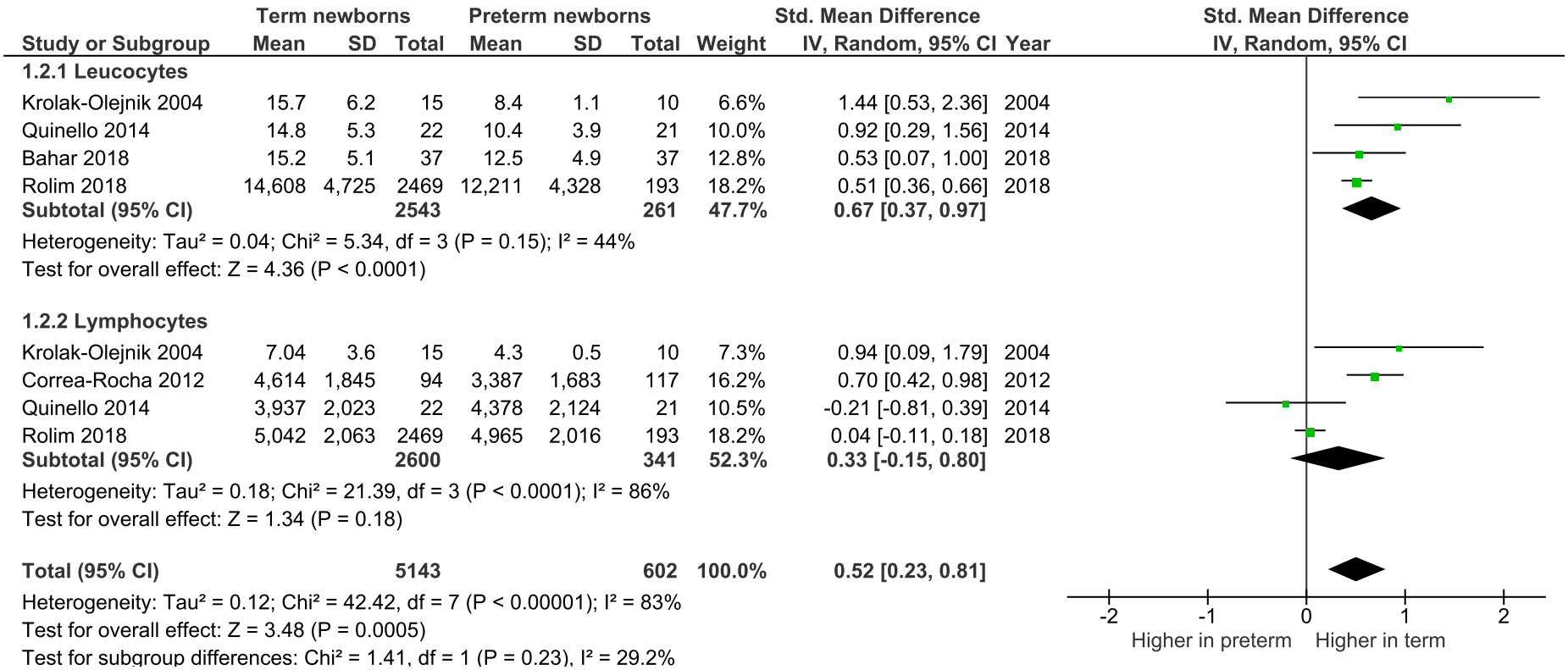

Figure 4: Forest plot of cord blood leucocytes and lymphocytes for term versus preterm birth. Means and SDs for Correa-Rocha *et al*. and Quinello *et al*. are estimated from means and quartiles. Quinello *et al*. included three exposure groups: moderately preterm, late preterm and term newborns; data for late preterm and term newborns were used for meta-analysis. SD, standard deviation; IV, inverse variance; CI, confidence interval.

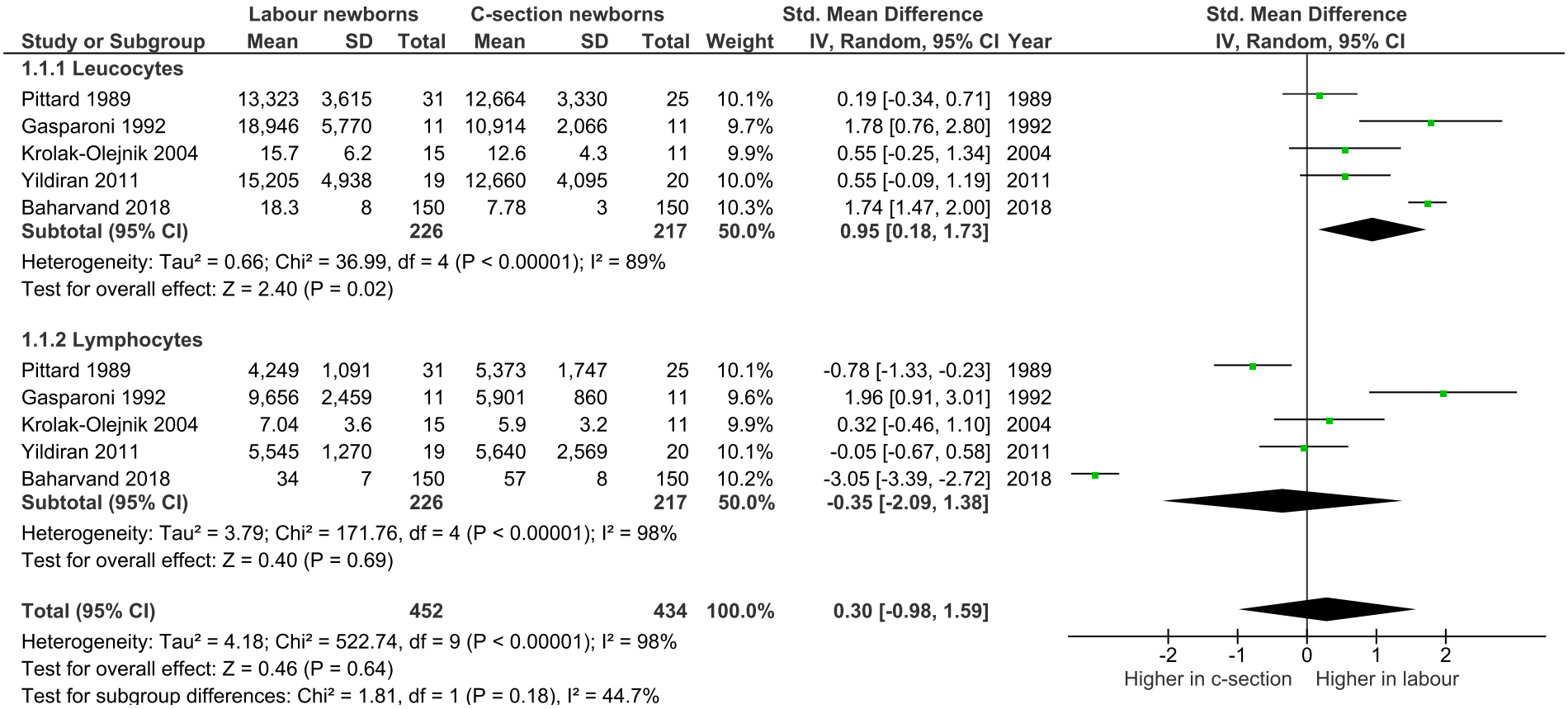

Figure 5: Forest plot of cord blood leucocytes and lymphocytes for labour versus c-section delivery. SD, standard deviation; IV, inverse variance; CI, confidence interval; c-section, caesarean section.

Discussion

We found term birth (compared to PTB) and labour (compared to c-section) were associated with increased leucocytes, but not lymphocytes, in cord blood. Study heterogeneity was higher for lymphocyte analyses compared to leucocyte analyses (I^2=86% and 98% versus 44% and 89%, respectively). It is possible cord lymphocytes increase with GA, but confounding led to underestimation of this relationship. Residual confounding is inherent in observational studies; consequently, even statistically significant meta-comparisons in this review were rated as “low” or “very low” according to the GRADE assessment.

To our knowledge, this is the first systematic review to assess the relationship between cord blood inflammatory cells and GA or MOD. Based on a small number of observational studies, increased numbers of cord blood leucocytes—but not lymphocytes—appear to be associated with term delivery and labour compared to preterm delivery and c-section, respectively. This may indicate maturation of the fetal immune system with advancing gestation (18). However, all studies used CV blood (or failed to specify, meaning it was likely venous). Hence, this could alternatively reflect increasing placental inflammation in preparation for labour. Future studies must sample the CA and CV to investigate potential differences in fetal and placental circulations. Therefore, we conducted a study (presented below) to further characterise the interactions between the fetus, placenta and mother, in addition to the role of lymphocytic FAAH.

Investigating the role of lymphocytes and fatty acid amide hydrolase in term human pregnancy

Materials and methods

This study aimed to investigate both i) T lymphocyte populations and ii) FAAH levels in IV (maternal), CA (fetal) and CV (placental) blood in women delivering via elective caesarean section (eCS) at term. We originally intended to compare these women to a cohort undergoing spontaneous labour (SL) and vaginal delivery, but as participant recruitment was delayed due to COVID-19 restrictions this was not achievable during the study period. Samples were collected from women with low-risk pregnancies delivering via eCS at Southmead Hospital Central Delivery Suite. Inclusion and exclusion criteria are listed in Table 5. Recruitment began in April 2021 and finished in May 2021. A total of *n*=13 women were recruited. Blood was taken from three sites for each participant:

1. CV
2. CA
3. Placental IV space, as described previously (19)

Inclusion criteria	Exclusion criteria
Singleton pregnancy	Multiple pregnancy
BMI 18 to 30	Chorioamnionitis
Age 20 to 40 years	Diabetes
	Pre-eclampsia
	Metabolic conditions
	Obstetric cholestasis
	BMI under 18 or over 30
	Age under 20 or over 40 years
	PTL
	IOL

Table 5: Inclusion and exclusion criteria for participant enrolment. BMI, body mass index; PTL, preterm labour; IOL, induction of labour.

Mononuclear cells (MNCs) were isolated within four hours by density gradient centrifugation (Fig. 6), cryopreserved and stored at -80^{o}C.

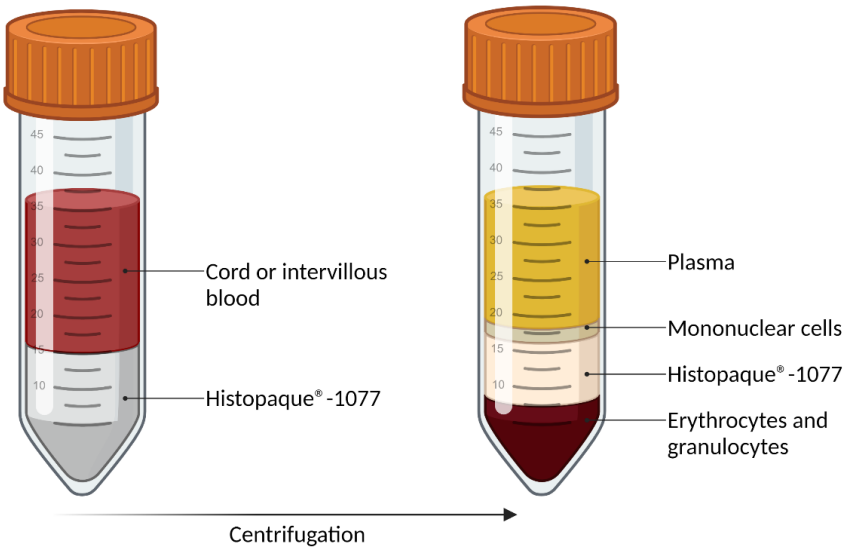

Figure 6: Procedure for density gradient centrifugation. The sample is layered over Histopaque®-1077 and centrifuged to separate the blood components according to their molecular weight. After discarding the upper plasma player, the interface MNC layer is aspirated using a sterile Pasteur pipette and stored in a new tube. Created with BioRender.com.

MNCs were thawed rapidly and stained with monoclonal antibodies for CD3, CD4, CD8, CD45, CD16, CD56, CD161 and TCR Vα7.2 (Table 6). Cells were acquired on a NovoCyte®.

Antigen	Conjugate	Clone
CD3	FITC	HIT3a
CD4	PE	OKT4
CD8	APC	SK1
CD45	PE/Dazzle™	HI30
CD16	PE/Cyanine7	3G8
CD56	Alexa Fluor® 700	HCD56
CD161	PerCP/Cyanine5.5	HP-3G10
TCR Vα7.2	Brilliant Violet 421™	3C10

Table 6: Antibodies for flow-cytometry analysis of cord and intervillous blood immune phenotypes. CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; PerCP, peridinin chlorophyll protein; TCR, T cell receptor.

Analysis was performed in FlowJo™. Compensation matrices were calculated using single-stained MNCs. Cells were identified based on forward and side scatter characteristics (Table 7).

Immune cell subset	Antigenic profile
Leucocytes	CD45+
T cells (all)	CD3+
CD4+ T cells	CD3+/CD4+
CD8+ T cells	CD3+/CD8+
MAIT cells	CD3+/CD8+/CD161+/TCR Vα7.2+
NK cells	CD3-/CD56^{bright}CD16^{dim} or CD3-/CD56^{dim}CD16^{bright}

Table 7: Antigenic definition of immune cell subsets for flow cytometry. CD, cluster of differentiation; MAIT, mucosal associated invariant T cells; TCR, T cell receptor.

MNCs were thawed and subjected to two rounds of quick-freezing and slow-thawing at room temperature to break cell membranes. Cell homogenates were then centrifuged and the supernatant used for FAAH protein quantification by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis was performed using GraphPad Prism version 9.1.2. Measures of central tendency are presented as means with standard error of the mean (SEM). Comparisons of two groups were performed using Welch's t-test; comparisons of multiple groups were performed using the one-way ANOVA with Tukey's post-hoc test. *P* values ≤0.05 were considered statistically significant.

Results

Upon quantifying FAAH in MNCs of CA, CV and IV blood from women undergoing eCS, only eight samples produced absorbance values within the linear range of detection. These comprised two CA, six CV and no IV samples, reflecting issues obtaining sufficient MNC counts from CA and IV blood. There was no significant difference in FAAH expression (standardised to MNC count) between CA and CV (Welch's t-test, *P*=0.63) (Fig. 7). These data indicate the MNC isolation technique for CA and IV blood requires further optimisation for meaningful comparison of FAAH expression between the three vascular compartments.

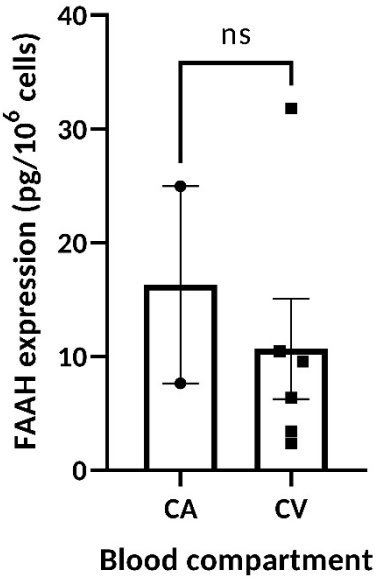

Figure 7: MNC FAAH expression in CA and CV blood. There was no significant difference in FAAH expression for MNCs in CA and CV blood (*P*=0.63). Data analysed by Welch's t-test. MNC, mononuclear cell; FAAH, fatty acid amide hydrolase; CA, cord artery; CV, cord vein.

We next analysed MNCs from CA, CV and IV blood by flow cytometry (Fig. 8). CD3+ cells were relatively increased in IV compared to CA and CV blood (one-way ANOVA, *P*=0.0045). There was a trend for a proportional decrease in CD4+ T cells (*P*=0.0751) and an increase in CD8+ T cells (*P*=0.1833) in IV compared to cord blood, although there was no significant difference in the CD4/CD8 ratio between the three compartments (*P*=0.5091) (Fig. 9). There were no significant differences in the proportion of MAIT cells between CA, CV and IV blood (Table 8). We were unable to analyse the flow cytometry data for NK subsets, potentially due to spectral overlap of the APC and Alexa Fluor® 700 fluorophores.

Subpopulation	Mean % ±SEM Cord artery (CA) blood	Cord vein (CV) blood	Intervillous (IV) blood	ANOVA, *P*	Tukey's test, *P*		
					CA vs CV	CA vs IV	CV vs IV
T cells (CD45+/CD3+) *	47.17±6.98	41.6±4.67	68.93±3.74	**0.0045**	0.743	**0.0248**	**0.0051**
CD4+ T cells (CD3+/CD4+) †	75.53±2.64	77.04±2.19	67.69±3.67	0.0751			
CD8+ T cells (CD3+/CD8+) †	21.83±2.21	20.84±2.04	27.07±2.99	0.1833			
CD4/CD8	3.78±0.54	4.02±0.58	3.00±0.78	0.5091			
MAIT cells (CD3+/CD8+/ CD161+/Vα7.2+) †	0.90±0.14	0.88±0.14	0.98±0.15	0.8681			

Table 8: Composition of immune cell subsets in CA, CV and IV blood, with extracellular markers used for identification by flow cytometry. Values are mean ± SEM; n=13. Data analysed by one-way ANOVA (with Tukey's post-hoc test, T cells only). CA, cord artery; CV, cord vein; IV, intervillous. *Frequency as a percentage of leucocytes. †Frequency as a percentage of T cells.

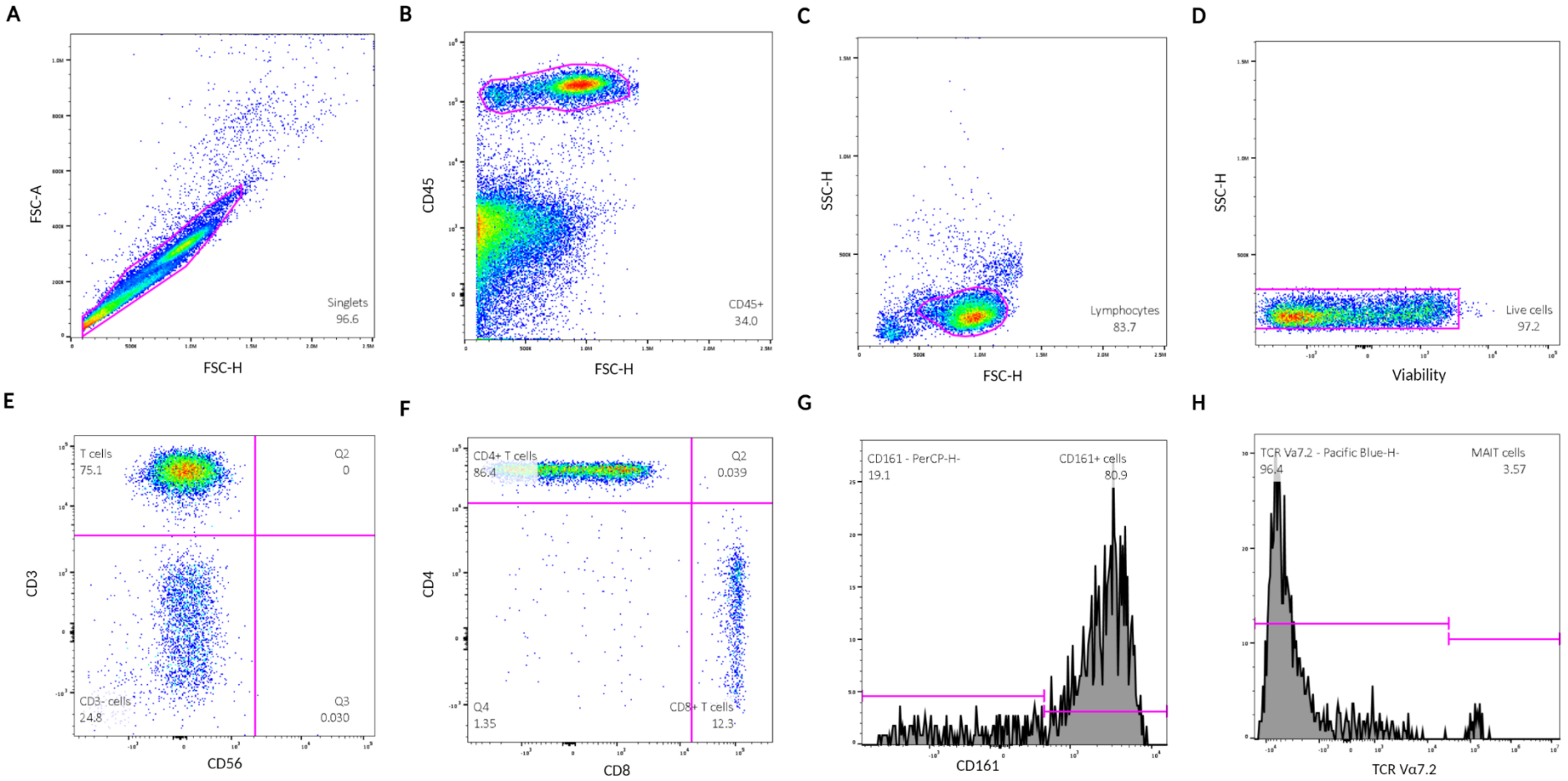

Figure 1: Representative flow cytometry plots of MNCs isolated from cord and maternal blood. **A.** Cells were initially gated on forward-side scatter properties. Singlets were identified on the basis of area versus height parameters of FSC. **B.** Leucocytes were separates were separated from monocytes, platelets and debris on the basis of height parameters of SSC versus FSC. **D.** Live cells were separated using a viability gate; cell viability was always above 95%. **E.** CD3+ T-cells were identified. **F.** CD4+ and CD8+ T-cell subsets were identified. **G.** The CD8+ T-cell population was selected and a CD161 gate was applied. **H.** The CD161+ population was selected and a TCR Vα7.2 gate was applied to identify MAIT cells. MNCs; mononuclear cells; RBCs, red blood cells; FSC, forward scatter; SSC, side scatter; MAIT, mucosal associated invariant T.

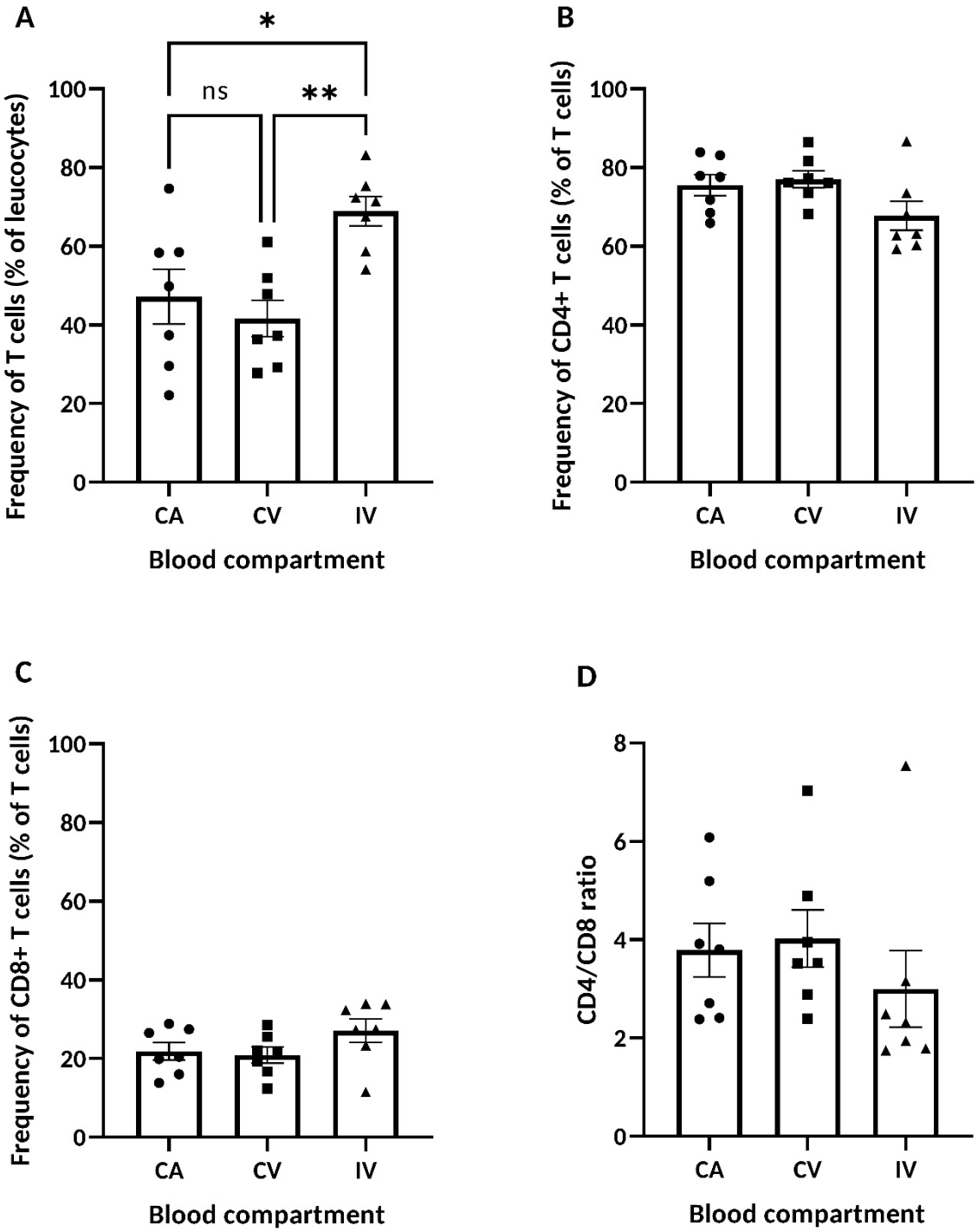

Figure 2: T cells are increased in maternal blood compared to cord blood. CD45+ cells were characterised by flow cytometry staining. **A.** The T cell population (as a percentage of leucocytes) is significantly higher in IV compared to CA and CV blood. There were no significant differences in CD4+ T cell or CD8+ T cell populations (as a percentage of T cells) between CA, CV and IV blood (**B–D**). Graphs show mean ± SEM; n=13. Data analysed by one-way ANOVA (**A-D**) with Tukey's Honestly Significant Difference post-hoc analysis (**A**). ns, $P>0.05$; * $P\leq0.05$; ** $P\leq0.01$. CA, cord artery; CV, cord vein; IV; intervillous.

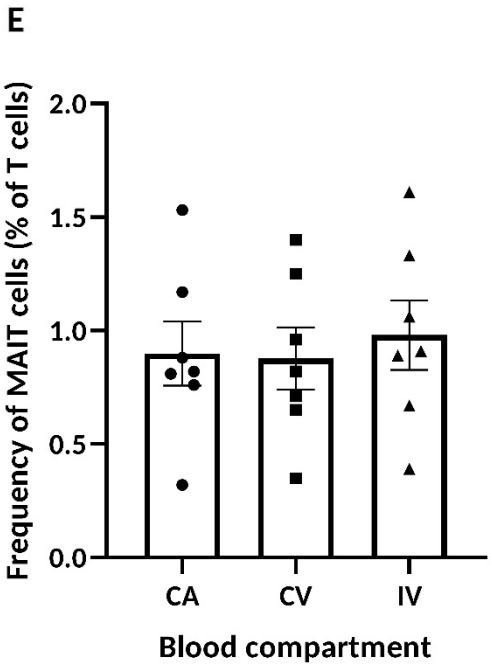

Figure 9 (continued). There was no significant difference in MAIT cell populations (as a percentage of T cells) between CA, CV and IV blood (**E**). Graph shows mean ± SEM; n=13. Data analysed by one-way ANOVA. CA, cord artery; CV, cord vein; IV; intervillous; MAIT, mucosal associated invariant.

Discussion

This study was predicated on a metabolomic study by Birchenall *et al*. which showed labour was associated with increased eCBs in cord blood (10). We therefore sought to investigate FAAH activity (and its lymphocytic regulation) in women undergoing eCS. We found T lymphocytes were increased in IV (maternal) compared to cord blood, while CA (fetal) and CV (placental) blood had comparable lymphocyte populations. Our study is unique as we sampled from CA (and CV) thus showing for the first time that it is possible to perform flow cytometric analysis of blood from this compartment, despite low collection volumes and sampling issues (9).

As AEA has been shown to be higher in CV compared to CA blood (14), we hypothesised that changing FAAH activity causes local production or transportation of eCBs across the placenta. While MNC isolation requires further refinement to obtain sufficient yields to compare FAAH levels in all three compartments, our data provide an indication of the minimum MNC numbers required for future assays.

Importantly, the T-lymphocytic composition of CA and CV blood were similar, which is perhaps unsurprising as these samples were taken from women in whom the trigger(s) for labour had not yet occurred. The Birchenall *et al*. study found the CA and CV had distinct metabolomic profiles with different modes of delivery and generated the hypothesis that immune cells in the uterus are involved in the trigger for SL (7, 10). Hence, it is plausible the immune phenotypes of CA and CV blood diverge during labour. Further research will seek to optimise the methods employed by this study and compare these results to those from women undergoing SL.

Word count: 1999 (excluding tables and figure legends)

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