Abnormal placental CD8\(^+\) T-cell infiltration is a feature of fetal growth restriction and pre-eclampsia

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Edited by: Laura Bennet & Suzanne Miller

Key points

- Placental pathological abnormalities are more frequently observed in complicated pregnancies than in healthy pregnancies.
- Infiltration of CD8\(^+\) T-cells into the placental villous tissue occurred in both fetal growth restriction and pre-eclampsia, whereas CD79\(\alpha^+\) B-cell infiltration was only apparent with reduced fetal growth.
- Vascularization, fibrin depositions, macrophage and neutrophil infiltration in the placenta did not differ between healthy and complicated pregnancies.

Abstract  Fetal growth restriction (FGR) and pre-eclampsia are severe, adverse pregnancy outcomes. Alterations in placental histology are frequently reported in these pregnancy complications and are often based upon scoring by pathologists. However, many alterations are also observed in placenta from uncomplicated pregnancies. Moreover, knowledge of disease state may bias assessment. We sought to perform an objective comparison of placental microscopic appearance in normal and complicated pregnancies. Placental villous tissue (\(n = 823\)) and edge biopsies (\(n = 488\)) from 871 individual, singleton pregnancies were collected after delivery. Cases of small-for-gestational age (SGA) or pre-eclampsia were matched with healthy controls. A subset of the SGA cases displayed signs of FGR. Cases of preterm delivery were also included. Tissue sections were stained with haematoxylin and eosin or antibodies for CD8, CD14, CD31, CD79\(\alpha\) and elastase. Images were scored by two experienced pathologists for pathological features or analysed by image analysis and stereology. Analyses were performed blind to case-control status.

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status and gestational age. Volume fraction of T-cells increased in placentas from pregnancies complicated by pre-eclampsia (adjusted odds ratio (aOR) 1.46, 95% CI: 1.12–1.90) and FGR (aOR 1.64, 95% CI: 1.11–2.43), whereas B-cells only increased in FGR (aOR 1.65, 95% CI: 1.05–2.60). Pathological abnormalities in villous tissue were reported in 21.4% (88/411) of complicated pregnancies and 14.3% (52/363) of controls (OR 1.62, 95% CI: 1.12–2.37). There were no differences in the fractions of endothelial cells, fibrin deposition, macrophages and neutrophils when comparing normal and complicated pregnancies. In conclusion, FGR and pre-eclampsia are associated with T-cell infiltration of the placenta and placental pathological abnormalities.

(Received 31 January 2020; accepted after revision 28 August 2020; first published online 4 September 2020)

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Introduction

Each year millions of pregnancies worldwide are complicated by fetal growth restriction (FGR) or pre-eclampsia. These pregnancy complications are associated with a higher risk of mortality and morbidity for both mother and baby (Lawn et al. 2014; Salam et al. 2014; Say et al. 2014; Mol et al. 2016). At present, our understanding of the aetiology of FGR and pre-eclampsia is incomplete. Yet, changes in placental histology are frequently reported in such pregnancy complications. The changes include placental lesions, which comprise chronic inflammatory lesions, massive fibrin deposition and maternal vascular malperfusion (Pathak et al. 2011; Kim et al. 2015; Devisme et al. 2017; Wright et al. 2017). Villitis of unknown aetiology (VUE) is one of the key placental inflammatory lesions (Kim et al. 2015). It has previously been shown in VUE that there is an infiltration of T-cells of maternal origin into villous tissue (Redline & Patterson, 1993; Kim et al. 2008). Other placental changes reported in association with pregnancy complications include reduced surface area of fetal capillaries and terminal villi (Egbor et al. 2006; Mayhew, 2009).

There are two main areas of concern in relation to some of the previous literature. First, the diagnosis of a placental pathology is made by a pathologist and defined subjectively. Although some studies have blinded comparisons of cases and controls, the majority do not. Importantly, knowing that a sample has come from a complicated pregnancy is likely to lead to ascertainment bias. Second, pre-eclampsia and FGR are associated with multiple maternal characteristics such as age, parity, body mass index (BMI) and smoking status. While epidemiological studies routinely account for potential confounding by such associated factors, many studies of both placental histopathology and stereology do not.

The aim of the present study was to investigate whether pregnancy complications are associated with alterations in placental histology while accounting for potential confounding factors. Therefore, we analysed a large number of placentas from well-phenotyped cases (FGR, preterm birth, pre-eclampsia) and controls. Cases were matched to a control based on potentially confounding maternal characteristics. Placental sections were stained for presence of B-cells, endothelial cells, macrophages, neutrophils and T-cells with specific antibodies; sections were also stained with haematoxylin and eosin (H&E) for observation of pathological features. The analysis was performed by image analysis, stereology and scoring by two pathologists. All analyses were carried out blinded to the case–control status, gestational age and maternal characteristics.

Methods

Ethical approval

The Pregnancy Outcome Prediction (POP) study was approved by the Cambridgeshire 2 Research Ethics Committee (reference number 07/H0308/163), and the study has previously been described (Pasupathy et al. 2008; Gaccioli et al. 2017). Briefly, 4212 nulliparous women with a viable singleton pregnancy were recruited at their dating scan (approx. 12 weeks’ gestation) in the Rosie Hospital, Cambridge, UK and followed through pregnancy until delivery. The participating women had serial phlebotomy and ultrasound scans during pregnancy, with placentas collected after delivery. The characteristics of eligible women and participating women in the POP study have previously been described (Sovio et al. 2015). All participants gave informed written consent for participation in the study and subsequent analyses of samples obtained. The POP study was conducted in accordance with the Declaration of Helsinki, according to the version current at the time of ethics submission (2004).

Patient selection

Cases of small-for-gestational age (SGA) or pre-eclampsia were matched to healthy controls. SGA was defined as a birth weight <5th percentile (based on customized birth weight; Gardosi et al. 1995). An infant was classified as
## Table 1. Patient characteristics of the women in the study sample by birth outcome

|                      | Control | SGA | PE | SGA and PE | Preterm |
|----------------------|---------|-----|----|------------|---------|
| **n**                | 406     | 170 | 219| 27         | 49      |
| **Maternal BMI (kg/m²)** | 24.4 (22.5, 27.3) | 24.7 (22.5, 27.7) | 27.0 (23.3, 31.9) | 27.3 (24.6, 31.2) | 24.7 (22.6, 26.3) |
| **Maternal age (years)** | 30.5 (27.3, 33.5) | 30.4 (26.7, 34.1) | 29.1 (25.4, 33.1) | 30.0 (23.0, 34.1) | 30.7 (27.9, 33.3) |
| **Gestational age (weeks)** | 40.1 (39.3, 41.0) | 40.1 (39.1, 41.3) | 39.9 (38.6, 40.7) | 37.4 (33.6, 39.0) | 34.4 (31.9, 35.4) |
| **Sample collection time (hours)** | 3.4 (0.2, 9.7) | 5.5 (0.5, 10.6) | 2.2 (0.2, 9.3) | 6.8 (0.3, 10.7) | 0.7 (0.2, 8.4) |
| **Fetal sex (female)** | 207 (51%) | 90 (53%) | 94 (43%) | 17 (63%) | 18 (36.7%) |
| **Smoking at booking (yes)** | 41 (10.1%) | 41 (24.1%) | 32 (14.6%) | 3 (11.1%) | 6 (12.2%) |
| **Age stopped FTE (years)** | 21.0 (18.0, 23.0) | 20.5 (18.0, 23.0) | 19.5 (17.0, 23.0) | 19.0 (18.0, 21.0) | 21.0 (18.0, 23.0) |
| **Maternal height (cm)** | 165 (161, 170) | 165 (160, 169) | 165 (160, 168) | 163 (160, 166) | 165 (160, 167) |
| **Deprivation quartile** | 1 (lowest) | 101 (24.9%) | 41 (24.1%) | 51 (23.3%) | 9 (33.3%) |
|                      | 2       | 92 (22.7%) | 30 (17.7%) | 58 (26.5%) | 2 (7.4%) |
|                      | 3       | 109 (26.9%) | 39 (22.9%) | 48 (21.9%) | 12 (44.4%) |
|                      | 4 (highest) | 84 (20.7%) | 49 (28.8%) | 56 (25.6%) | 2 (7.4%) |
| **Missing** | 20 (4.9%) | 11 (6.5%) | 6 (2.7%) | 2 (7.4%) | 0 (0%) |
| **Ethnicity** | Non-white | 21 (5.2%) | 10 (5.9%) | 7 (3.2%) | 0 (0%) |
|                      | White | 380 (93.6%) | 157 (92.4%) | 210 (95.9%) | 27 (100%) |
|                      | Missing | 5 (1.2%) | 3 (1.8%) | 2 (0.9%) | 0 (0%) |
|                      | Married (yes) | 285 (70.2%) | 104 (61.2%) | 141 (64.4%) | 17 (63.0%) |
|                      | Any alcohol consumption | 18 (4.4%) | 6 (3.5%) | 6 (2.7%) | 0 (0%) |
| **Type I or type II diabetes** | 0 (0%) | 1 (0.6%) | 5 (2.3%) | 0 (0%) | 1 (2.0%) |
| **UtA Doppler mean PI (highest decile)** | 26 (6.4%) | 42 (24.7%) | 36 (16.4%) | 16 (59.3%) | 3 (6.1%) |
| **Missing** | 10 (2.5%) | 5 (2.9%) | 7 (3.2%) | 0 (0%) | 4 (8.2%) |
| **Birth weight (g)** | 3500 (3265, 3685) | 2690 (2385, 2860) | 3400 (3090, 3770) | 2180 (1600, 2390) | 2260 (1825, 2520) |
| **Induction of labour** | 97 (23.9%) | 54 (37.8%) | 137 (62.6%) | 10 (37.0%) | 0 (0%) |
| **Mode of delivery** | Vaginal | 264 (65.0%) | 125 (73.5%) | 122 (55.7%) | 10 (37.0%) |
|                      | Intrapartum | 93 (22.9%) | 21 (12.6%) | 70 (30.2%) | 3 (11.1%) |
|                      | Caesarean | 49 (12.1%) | 24 (14.1%) | 27 (12.3%) | 14 (51.9%) | 0 (0%) |

Median (interquartile range) or number (%) are given as appropriate. Smoking, maternal age and BMI were recorded at the booking appointment (~12 weeks of gestation) and other maternal characteristics were obtained from the 20-week questionnaire. Preterm includes spontaneous onset of labour before 37 weeks’ gestation. BMI, body mass index; FTE, full-time education; PE, pre-eclampsia using the ACOG 2013 definition; PI, pulsatility index; SGA, small-for-gestational age using customized reference centile; UtA, uterine artery.

FGR if the newborn had a birth weight <5th percentile together with one or more of the following abnormal ultrasonic features: abnormal uterine or umbilical cord Doppler, or low abdominal circumference growth velocity, as previously described (Sovio et al. 2015; Cleaton et al. 2016). The American Congress of Obstetricians and Gynecologists 2013 Guidelines were used to define pre-eclampsia and severe pre-eclampsia (ACOG, 2013). Healthy controls had uncomplicated pregnancies, with no evidence of pre-existing or pregnancy-induced hypertension or diabetes, normal fetal growth (i.e. delivery of an infant with a customized birth weight percentile in the normal range: 20–80th centile) (Gardosi et al. 1995), and no evidence of obstetric complications. Cases and controls were matched using the following criteria: mode of delivery (absolute match), as close as possible maternal BMI, age of the mother, gestational age at delivery, sample collection time, maternal smoking and fetal sex. Preterm cases were defined as spontaneous deliveries between ≥24 weeks’ gestation (wkGA) and...
<37 wkGA, with no pre-eclampsia. Preterm cases were compared with the controls described above, but limited to the pregnancies with a spontaneous delivery at term (≥37 wkGA). In total, samples from 871 placentas were analysed and the patient characteristics of cases and controls are presented in Table 1. Included in the patient characteristics are self-reported ethnicity and marital status, as well as deprivation quartile, which was based on the Index of Multiple Deprivation 2007 (Noble et al. 2011). Socio-demographic factors, such as marital status, as well as the villous tissue in one tissue section. Tissue was collected from areas with visible damage, hematomas, or infarctions. To remove maternal blood, the biopsies were washed in ice-cold phosphate-buffered saline. The tissue samples were fixed in a 10% formalin solution, neutral buffered (cat. no. HT501128; Sigma-Aldrich, Gillingham, UK) for 24 h (4°C). After fixation, the four villous tissue samples were embedded into one paraffin wax block and the edge biopsy embedded into a separate paraffin wax block. The orientation of the villous tissue biopsies was random.

Histological staining

Tissue sections of the placental tissue were cut (3 µm) onto SuperFrost Plus glass slides (cat. no. 631-0108; VWR, Lutterworth, UK). Staining of placental villous tissue sections was carried out on a clinical-grade automated IHC (immunohistochemistry) stainer (BOND-III; Leica Biosystems, Wetzlar, Germany) with the BOND Polymer Refine Detection kit (cat. no. DS9800; Leica Biosystems). The antibodies used are described in Table 2. H&E staining of the placental villous tissue sections was performed on a Leica Autostainer XL (Leica Biosystems, Wetzlar, Germany) with VFM Harris Haematoxylin (acidified, cat. no. RBA-4202-00A; CellPath, Newtown, UK) and Eosin Y Stain (1% aqueous, cat. no. RBC-0100-00A; CellPath). The placental edge biopsies were stained manually with eosin (1% aqueous; cat. no. PRC/66/1; Pioneer Research Chemicals, Colchester, UK) and haematoxylin (Mayer’s, cat. no. PRC/R/42; Pioneer Research Chemicals). After staining, all slides were scanned with an Aperio AT2 scanner (×20 magnification; Leica Biosystems). The cutting, staining and scanning of samples was carried out in batches. Matched cases and controls were processed together, in parallel, in the same batch.

Image analysis

Image analysis was performed using Visiopharm Integrator System (version 6.2; Visiopharm, Hoersholm, Denmark). The regions of interest (ROI) were outlined using the software’s automatic ROI identifier, with any subsequent staining artefacts, dust, etc. manually excluded. To quantify total tissue area, area positive for 3,3-diaminobenzidine (DAB) staining and hotspots of DAB staining (see below for definition), automatic applications containing multistep algorithms were developed. In the pre-processing step, the following features were identified: DAB staining, intervillous space, nuclei, tissue and areas of calcification. The subsequent processing steps involved excluding the intervillous space and determination of criteria for eliminating small areas by a lower cut-off: DAB (artefacts, ≤15 µm²), nuclei (≤10 µm²), tissue (≤100 µm²) and areas of calcification (≤100 µm²). Total tissue area (µm²), area of DAB stain (µm²) and proportion of DAB positive area over total tissue area based on the classified image were all calculated. In order to adjust for possible batch variability in staining intensity, the application was trained to recognize all the features (DAB, intervillous space, nuclei, tissue and calcification) on a montage containing images from all slides in each batch. The trained application was then run on the whole slide, for all slides within the batch. The proportion of B-cells, macrophages, neutrophils, and T-cells over total tissue area was calculated for each organ.

An automatic application to detect areas of accumulation for DAB positive features (referred to as hotspots) was developed utilizing the classified image. The automatic application for hotspots used the software’s heatmap generating function. The heatmap was based on the image analysis of each slide, and the result was classified into a binary variable of hotspots present or absent. The image analysis was performed blind to case or control status by S.L., M.L., E.C. and C.Y.

Stereology

Stereology was performed using Visiopharm Integrator System (version 5.2). ROI were drawn closely around all four villous tissue samples. The analysis was performed with point probes and meander sampling to estimate volume densities within the placenta of fibrin and
Table 2. Antibodies used in study

| Primary antibody | Negative control | Antigen retrieval (minutes) | Primary antibody dilution |
|------------------|------------------|-----------------------------|---------------------------|
| CD8 (clone CB/144B) | M7103 (Agilent Technologies LDA UK Ltd, Cheadle, UK) Mouse negative control (cat. no. PA0996; Leica Biosystems) | H1 (30) | 1:50 |
| CD14 (clone 7) | NCL-CD14-223 (Leica Biosystems) Mouse IgG2a control (ab18414; Abcam, Cambridge, UK) | H2 (20) | 1:100 |
| CD31 (clone JC70A) | M082301-2 (Agilent Technologies LDA UK Ltd) Mouse negative control (cat. no. PA0996; Leica Biosystems) | E1 (10) | 1:50 |
| CD79α (clone JCB117) | M705001-2 (Agilent Technologies LDA UK Ltd) Mouse negative control (cat. no. PA0996; Leica Biosystems) | H1 (30) | 1:50 |
| Elastase (clone NP57) | M0752 (Agilent Technologies LDA UK Ltd) Mouse negative control (cat. no. PA0996; Leica Biosystems) | None | 1:100 |

*Antigen retrieval: E1, Bond Enzyme Pre-treatment Kit (cat. no. AR9551; Leica Biosystems); H1, pH6, Bond Epitope Retrieval 1 (cat. no. AR9961; Leica Biosystems), H2, pH9, Bond Epitope Retrieval 2 (cat. no. AR9640; Leica Biosystems).

endothelial cells (CD31 positive cells). Approximately 30 fields of view per placenta (distributed across the four villous tissue samples) were analysed. The proportion of endothelial cells or fibrin over total tissue area was calculated for each placenta. The stereology analysis was performed blind to case or control status by E.E.

Pathologist scoring

Histopathological examination of H&E stained villous tissue and edge biopsies was carried out by two experienced placental pathologists (F.J. and N.S.), blind to case or control status, gestational age and maternal characteristics. The classification of any histological features was performed according to previous agreed and standardized criteria (Table 3) (Redline, 2015; Turowski & Vogel, 2018). Pathological features were recorded qualitatively (present or absent). In cases with more than one placental lesion present, each lesion was documented. The histopathological scoring was carried out independently by the two pathologists; discrepancies between the classifications were resolved by consensus, still blind to case or control status.

Statistics

For data from the image analysis and stereology, the mean proportion for the four villous tissue biopsies for a given stain was calculated. This mean was used in the statistical analysis. It was transformed to improve normality of the distribution and used as a continuous variable in a conditional logistic regression model. Associations were expressed as odds ratio (OR) with 95% confidence interval for case status for a 1 standard deviation increase in the transformed placental proportion for given cell type, with adjustment for the maternal characteristics used in the matching of cases and controls (since matching does not eliminate confounding; Pearce, 2016). Prior to the adjusted analysis, missing values in maternal characteristics were imputed using the mean (continuous) or the most common value (categorical) in the whole POP study cohort. For descriptive purposes, median and interquartile range (IQR) of the untransformed proportion was reported by case–control status. Additionally, the mean percentage difference of the untransformed proportion between matched cases and controls was reported with 95% CI.

Differences in histological features between complicated (SGA, FGR, pre-eclampsia and severe pre-eclampsia) and healthy pregnancies as scored by the two pathologists were evaluated by conditional logistic regression. Differences between preterm cases and their controls were analysed using logistic regression. Data are presented as odds ratios (95% CI) and P-values. The associations between the presence of CD8+ T-cell hotspots and VUE was assessed separately in controls and cases by calculating the OR (95% CI) and Fisher's exact test P-value. All statistical analysis was performed using Stata, v15 (StataCorp, College Station, TX, USA).

Results

Histological staining of placental villous tissue

For the image analysis and stereology, we used four villous biopsies from 775 women stained with H&E and five different antibodies. Therefore, the current examination by image analysis and stereology is based on quantification of over 18,000 different stained samples (a small number of biopsies were excluded from the analysis due to technical issues). The proportion of tissue positively stained for each of the cellular markers in placentas from pre-eclampsia,
The proportion of CD8$^+$ T-cells (Fig. 3A and B) was higher in placental tissue from pregnancies complicated by pre-eclampsia compared to controls (adjusted OR (aOR) 1.46, 95% CI: 1.12–1.90; $P = 0.0048$; $n = 482$). Proportion of T-cells was also higher in SGA compared to control (aOR 1.44, 95% CI: 1.11–1.86; $P = 0.0060$; $n = 370$). These associations were still present when limiting the analysis to cases with severe pre-eclampsia (aOR 2.05, 95% CI: 1.26–3.34; $P = 0.0038$; $n = 238$) and FGR (aOR 1.64, 95% CI: 1.11–2.43; $P = 0.0130$; $n = 211$). In 48 placentas, areas with accumulation of CD8 positive cells (hotspots) were detected (see FGR case in Fig. 2A). Presence of T-cell hotspots was associated with SGA (OR 6.33, 95% CI: 1.87–21.40; $P = 0.0030$; $n = 370$). An increased prevalence of hotspots in FGR placentas was not observed, but the confidence intervals were wide and overlapped with SGA (OR 3.00, 95% CI: 0.81–11.08; $P = 0.0994$; $n = 211$).

SGA, FGR and matched controls is presented in Table 4. For descriptive purposes, the mean percentage differences in proportion of positively stained areas between matched cases and controls are presented in Fig. 1. Statistical analysis describing differences between cases and control are presented in subsequent sections. Selected images of B-cells, endothelial cells, H&E, macrophages, neutrophils and T-cells from an FGR case are shown in Fig. 2.
Pre-eclampsia was not associated with increased presence of hotspots (OR 0.69, 95% CI: 0.30–1.62; \( P = 0.3964; n = 482 \)).

**Macrophages**

The proportion of CD14\(^+\) macrophages (Fig. 3A and C) in the placenta did not differ between healthy and complicated pregnancies. Macrophages in the placenta were similar between pre-eclampsia and controls (aOR 1.00, 95% CI: 0.67–1.50; \( P = 0.9944; n = 480 \)), or SGA pregnancies and controls (aOR 1.06, 95% CI: 0.70–1.59; \( P = 0.7934; n = 378 \)).

**B-cells**

The proportion of CD79\(\alpha\)^+ B-cells (Fig. 3A and D) did not differ notably between cases of pre-eclampsia compared to healthy controls (aOR 1.24, 95% CI: 0.87–1.77; \( P = 0.2285; n = 430 \)). In contrast, the proportion of B-cells was higher in pregnancies complicated by SGA than in controls (aOR 1.54, 95% CI: 1.13–2.10; \( P = 0.0069; n = 370 \)) and this was also observed in FGR placentas (aOR 1.65, 95% CI: 1.05–2.60; \( P = 0.0289; n = 211 \)). In 220 placentas, hotspots of areas with accumulation of CD79\(\alpha\) positive cells were detected. The incidence of B-cell hotspots was more prevalent in SGA placentas (OR 1.64, 95% CI: 1.09–2.47; \( P = 0.0181; n = 370 \)) and FGR placentas (OR 1.97, 95% CI: 1.13–3.43; \( P = 0.0164; n = 211 \)) compared to matched controls.

**Neutrophils**

There was no difference in the proportion of neutrophils (elastase positive tissue; Fig. 4A and B) in placentas when comparing pre-eclampsia with controls (aOR 1.15, 95% CI: 0.83–1.58; \( P = 0.4030; n = 476 \)) or SGA pregnancies with controls (aOR 0.94, 95% CI: 0.67–1.31; \( P = 0.6997; n = 374 \)). With pre-eclampsia, neutrophil hotspots were somewhat more prevalent but the association was consistent with null (OR 2.25, 95% CI: 0.98–5.17; \( P = 0.0563; n = 474 \)).
**Endothelial cells**

The proportion of endothelial cells was assessed by measuring CD31 positive tissue (Fig. 4A and C). About one-third of the villous tissue stained positive for endothelial cells. There was no difference between pre-eclampsia and controls (aOR 1.31, 95% CI: 0.99–1.73; $P = 0.0560; n = 484$), or SGA pregnancies and controls (aOR 1.00, 95% CI: 0.74–1.34; $P = 0.9906; n = 378$).

**Fibrin**

Deposition of fibrin was quantified by stereological analysis of the H&E stained sections (Fig. 4A and D).

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**Figure 2. High magnification of histology sections**

Staining of placental tissue for T-cells (CD8), macrophages (CD14), endothelial cells (CD31), B-cells (CD79α), neutrophils (elastase) and H&E. Scale bar: 50 μm.
Placentas from pregnancies complicated by SGA had more fibrin deposits compared to placentas from healthy pregnancies (aOR 1.31, 95% CI: 1.00–1.71; \( P = 0.0498; n = 373 \)). There was no difference in fibrin deposits between placentas from pregnancies complicated by pre-eclampsia and controls (aOR 0.88, 95% CI: 0.68–1.12; \( P = 0.2991; n = 474 \)).

**Pathologist scoring**

For the pathologist scoring, one placental edge biopsy from 488 pregnancies and four placental villous tissue biopsies from 823 pregnancies were included. In total for the scoring, 871 unique placentas were included and almost 3800 H&E stained tissue biopsies were analysed. Selected images from an FGR case with matched control. Cells positive for the cellular marker shown in brown. Scale bar: 200 µm. B–D, adjusted odds ratios (95% CI) for the association of T-cells, macrophages or B-cells with pre-eclampsia (PE), severe PE, SGA and FGR. Odds ratios are given for 1 standard deviation increase in the transformed variable. The transformations were logarithmic (CD8) or square root (CD14 and CD79α). The models were adjusted for clinical characteristics matching variables by logistic regression.
examples of the staining/placental lesions are shown in Fig. 5.

In the villous tissue, signs of any placental lesion were more prevalent in complicated pregnancies than in healthy pregnancies (OR 1.63, 95% CI: 1.12–2.37; \( P = 0.0110; n = 774 \)). This increased prevalence of placental abnormalities was also evident when complications were separated into pre-eclampsia (OR 1.70, 95% CI: 1.01–2.84; \( P = 0.0446; n = 482 \)), SGA (OR 2.06, 95% CI: 1.21–3.51; \( P = 0.0080; n = 378 \)) and FGR (OR 1.94, 95% CI: 1.04–3.62; \( P = 0.0368; n = 217 \)). Specifically, maternal vascular malperfusion was more common in all pregnancy

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**Figure 4. Neutrophils, endothelial cells and fibrin in placental villous tissue**

A, selected images from a pre-eclampsia case with matched control. Cells positive for the cellular marker shown in brown. Scale bar: 200 µm. B–D, adjusted odds ratios (95% CI) for the association of neutrophils, endothelial cells, or fibrin deposits with pre-eclampsia (PE), severe PE, SGA and FGR. Odds ratios were adjusted for clinical characteristics matching variables by logistic regression. Odds ratios are given for 1 standard deviation increase in the transformed variable. The transformations were logarithmic (fibrin), square root (elastase) or no transformation (CD31). The models were adjusted for clinical characteristics matching variables by logistic regression.

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complications (OR 3.00, 95% CI: 1.34–6.71; $P = 0.0076; n = 774$), as well as when separated into pre-eclampsia (OR 4.00, 95% CI: 1.13–14.17; $P = 0.0317; n = 482$), severe pre-eclampsia (OR = 8.00, 95% CI: 1.00–63.96; $P = 0.0499; n = 238$) and SGA (OR 3.53, 95% CI: 1.17–10.67; $P = 0.0254; n = 378$). VUE was observed more often in pregnancy complications (OR 1.95, 95% CI: 1.09–3.49; $P = 0.0236; n = 774$), but VUE was only associated with SGA (OR 2.57, 95% CI: 1.14–5.80; $P = 0.0227; n = 378$) when the pregnancy complications were divided up into sub-groups. The pathological classification of VUE was strongly positively associated with the detection of CD8$^+$ T-cell hotspots. Among controls, the prevalence of VUE was 28% ($n = 5/18$) and 4% ($n = 13/343$) in the presence and absence of hotspots, respectively (OR 9.8, 95% CI: 3.4–30.5; $P = 0.0010$).

**Figure 5. Placental histological pathologies**

A, a slide with the four villous tissue biopsies from one placenta. The slide also contains a circular tonsil biopsy (dense, darker tissue; included as a positive control for T-cell and B-cell staining). Scale bar: 3 mm. B, edge tissue biopsy from a placenta. Scale bar: 2 mm. C, calcification of placental villous tissue. Scale bar: 200 μm. D, VUE. Scale bar: 200 μm. E, choriangioma. Scale bar: 100 μm. F, intervillous thrombus. Scale bar: 100 μm.
Table 5. Pathologist scoring of placental villous tissue

| Class                        | Pregnancy complication| Pre-eclampsia | Severe pre-eclampsia | SGA      | FGR      |
|------------------------------|-----------------------|---------------|----------------------|----------|----------|
| Abnormal*                    | 1.63 (1.12–2.37)      | 1.70 (1.01–2.84) | 2.00 (0.97–4.12)     | 2.06 (1.21–3.51) | 1.94 (1.04–3.62) |
|                              | P = 0.0110            | P = 0.0446     | P = 0.0605           | P = 0.0080 | P = 0.0368 |
| Fetal thrombotic vasculopathy | 0.66 (0.15–2.97)      | 0.67 (0.11–3.99) | NA                  | 0.84 (0.12–6.12) | NA       |
|                              | P = 0.5880            | P = 0.6569     |                     | P = 0.8659 |          |
| Immature                     | 0.82 (0.41–1.66)      | 0.86 (0.29–2.55) | 1.00 (0.25–4.00)    | 0.79 (0.34–1.85) | 0.94 (0.35–2.52) |
| Maternal vasculature malperfusion | 3.00 (1.34–6.71)    | 4.00 (1.13–14.17) | 8.00 (1.00–63.96)   | 3.53 (1.17–10.67) | 6.08 (0.74–49.77) |
|                              | P = 0.0076            | P = 0.0317     | P > 0.99            | P = 0.5927 | P = 0.8998 |
| Villitis of unknown aetiology | 1.95 (1.09–3.49)      | 1.91 (0.92–3.96) | 2.20 (0.76–6.33)    | 2.57 (1.14–5.80) | 1.83 (0.77–4.31) |
|                              | P = 0.0236            | P = 0.0823     | P = 0.0499          | P = 0.0254 | P = 0.0926 |

Odds ratios (95% CI) and P-value for the unadjusted measurements. *All pathological classifications combined (a placenta was classified as abnormal if it displayed signs of one or more of the pathologies listed in Table 3). † All pregnancy complications combined, including both pre-eclampsia and SGA. Analysis: conditional (fixed-effects) logistic regression of matched cases and controls was used, except for pregnancy complication as a composite outcome ordinary logistic regression was used. For some diagnoses, too few placentas with the pathology were observed for statistical analysis. Villous tissue analysis based on n = 774 (pregnancy complication), n = 482 (pre-eclampsia), n = 238 (severe pre-eclampsia), n = 378 (SGA), or n = 217 (FGR). NA: no analysis done; the models do not converge. No cases of severe pre-eclampsia with fetal thrombotic vasculopathy.

In the placental edge biopsies, signs of any placental lesion were associated with pregnancy complications (OR 1.66, 95% CI: 1.15–2.41; P = 0.0072; n = 465). This was also evident for the subgroups of pregnancy complications: pre-eclampsia (OR 1.63, 95% CI: 1.01–2.63; P = 0.0458; n = 298), severe pre-eclampsia (OR 2.00, 95% CI: 1.03–3.89; P = 0.0413; n = 148) and SGA (OR 2.25, 95% CI: 1.14–4.44; P = 0.0194; n = 194). When lesions were separated into individual groups (i.e. calcifications, chorangiosis, fibrin, immature, maternal vascular malperfusion, VUE), there were no substantial differences in the prevalence between complicated and healthy pregnancies (Table 6).

When comparing spontaneous preterm delivery (without pre-eclampsia or SGA) with healthy, spontaneous term deliveries, the only placental histological classification more prevalent in the preterm group was placental immaturity, and this was observed in both the villous biopsies (OR 3.98, 95% CI: 1.48–10.69; P = 0.0062; n = 263) and placental edge biopsies (OR 20.85, 95% CI: 5.34–81.44; P < 0.0001; n = 166). There was no difference in prevalence of abnormal features, maternal vasculature malperfusion or calcification (Table 7).

Discussion

In this study we quantified the placental proportion of leukocytes, endothelial cells and fibrin deposits in a cohort of cases of SGA or pre-eclampsia, matched with healthy controls. Placentas from pregnancies complicated by pre-eclampsia or FGR exhibited greater proportions of T-cells, more often displaying pathological abnormalities (such as maternal vasculature malperfusion) compared with controls. In cases of FGR, the placentas also contained higher proportions of B-cells, fibrin deposits and VUE. There was no difference in proportions of endothelial cells, macrophages or neutrophils in the villous tissue when comparing placenta from complicated and healthy pregnancies.

We observed increased placental levels of CD8+ T-cells in pregnancies complicated by pre-eclampsia, FGR and SGA. The infiltration was evident as an overall increase, but also in the case of reduced fetal growth there were more localized foci of T-cell hotspots. This observation was consistent with the increased reporting of VUE in placentas from SGA pregnancies by pathologist scoring. Together this suggests that placentas of complicated pregnancies display signs of inflammation, but that the pattern of inflammation and potential underlying cause...
Placental pathology and leukocytes in pre-eclampsia and FGR

Table 6. Pathologist scoring of placental edge biopsies

| Class                                | Pregnancy complication† | Pre-eclampsia | Severe pre-eclampsia | SGA      | FGR      |
|--------------------------------------|-------------------------|--------------|----------------------|----------|----------|
| Abnormal†                            | 1.66 (1.15–2.41)        | 1.63 (1.01–2.63) | 2.00 (1.03–3.89)     | 2.25 (1.14–4.44) | 2.25 (0.98–5.17) |
| P = 0.0072                           | P = 0.0458              | P = 0.0413   | P = 0.0194           | P = 0.0563 |
| Calcification                        | 0.94 (0.56–1.59)        | 1.05 (0.58–1.90) | 1.44 (0.62–3.38)     | 0.73 (0.29–1.81) | 0.75 (0.17–3.35) |
| P = 0.8246                           | P = 0.8788              | P = 0.3964   | P = 0.4931           | P = 0.7064 |
| Chorangiosis                         | 1.39 (0.23–8.38)        | 1.00 (0.14–7.10) | 1.00 (0.06–15.99)    | NA       |
| P = 0.7214                           | P > 0.99                | P > 0.99     | P > 0.99             | P > 0.99 |
| Fibrin                               | 1.09 (0.48–2.50)        | 0.78 (0.29–2.09) | 1.00 (0.25–4.00)     | 1.20 (0.37–3.93) | 2.50 (0.49–12.89) |
| P = 0.8307                           | P = 0.6180              | P > 0.99     | P > 0.99             | P > 0.99 |
| Immature                             | 0.92 (0.36–2.36)        | 1.20 (0.37–3.93) | 0.50 (0.09–2.73)     | NA       |
| P = 0.8596                           | P = 0.7633              | P > 0.99     | P > 0.99             | P > 0.99 |
| Maternal vasculature malperfusion     | 1.57 (0.92–2.68)        | 1.89 (0.84–4.24) | 2.4 (0.85–6.81)     | 1.90 (0.44–2.51) | 2.50 (0.49–12.89) |
| P = 0.1003                           | P = 0.1229              | P > 0.99     | P > 0.99             | P > 0.99 |
| Villitis of unknown aetiology         | 2.86 (0.91–8.99)        | 2.67 (0.71–10.05) | 1.67 (0.40–6.97)     | 4.00 (0.18–22.06) | 2.00 (0.04–10.00) |
| P = 0.0728                           | P = 0.1474              | P = 0.4843   | P = 0.2150           | P = 0.5714 |

Odds ratios (95% CI) and P-value for the unadjusted measurements. †All pathological classifications combined (a placenta was classified as abnormal if it displayed signs of one or more of the pathologies listed in Table 3). ‡All pregnancy complications combined, including both pre-eclampsia and SGA. Analysis: conditional (fixed-effects) logistic regression of matched cases and controls was used, except for pregnancy complication as a composite outcome ordinary logistic regression was used. For some diagnoses, too few placentas with the pathology were observed for statistical analysis. Placental edge biopsy analysis based on n = 465 (pregnancy complication), n = 298 (pre-eclampsia), n = 148 (severe pre-eclampsia), n = 194 (SGA) or n = 116 (FGR). NA: no analysis done; the models do not converge. No cases of FGR with chorangiosis or immature features.

may differ depending on the complication. Our finding is consistent with previous reports that complicated pregnancies are associated with placental inflammation (Kim et al. 2015). It has previously been described that VUE consists of confined infiltration of T-cells of maternal origin into placental villi (Redline & Patterson, 1993; Kim et al. 2008). Although we have not investigated the origin of the CD8+ T-cells detected, here we do show in a larger sample set that these changes are present and cannot be attributed to ascertainment bias or confounding by associated maternal characteristics.

There are several potential underlying causes for the CD8+ T-cell infiltration into the placenta. Potentially, infection by a pathogen could result in the recruitment of T-cells into the tissue. However, this is an unlikely cause of inflammation for the placentas investigated in the present study. We have previously shown that eukaryotic pathogens and bacteria are very rarely found in human placenta (Lager et al. 2018; de Goffau et al. 2019).

Placental oxidative stress is a feature of FGR and pre-eclampsia (Burton & Jauniaux, 2018; Burton et al. 2019). Potentially, this could contribute to T-cell infiltration. Blood in the intervillous space of the placenta contains more CD8+ T-cells than maternal peripheral blood (Solders et al. 2019a). Blood in the intervillous space also contains higher levels of chemokines, including Macrophage migration inhibitory factor (MIF), which attracts T-cells (Solders et al. 2019a). Since hypoxia causes an upregulation of MIF levels for human placenta in vitro (Ietta et al. 2007), it is therefore possible that the increase in number or their activation state may contribute to T-cell infiltration. However, this would not explain the presence of localized hotspots of T-cells, but rather an overall infiltration.

Another possibility is that the increased infiltration of CD8+ T-cells is an immunological response. Such an explanation has previously been suggested. In such a scenario, T-cell infiltration is causally associated with an adverse outcome, reflecting immune rejection of the fetus by the mother (Kim et al. 2015).

In this study, we have not determined whether the CD8+ T-cells are of fetal or maternal origin. Although T-cells detected in localized hotspots may be of maternal origin, in line with previous reports (Redline & Patterson,
neutrophils were not associated with complications during placentas, remains to be determined.

chemokine are involved in the B-cell infiltration of FGR et al 

CCL20 levels are increased by inflammatory signalling 

The placenta appears to occur in pregnancies complicated in the third trimester for pregnancies complicated by FGR (Xiong 1993; Kim et al. 2008), it is most likely we are measuring fetal and maternal CD8\(^+\) T-cells. Recent work has highlighted the mixed origins of cells in placenta, with the organ containing cells from both mother and child (Vento-Tormo et al. 2018; Pique-Regi et al. 2019).

Our CD79a\(^+\) B-cell findings further support an interpretation that placental inflammation differs in FGR and pre-eclampsia (as levels were elevated in the former but not for the latter). The origin of the placental B-cells remains to be determined, but maternal levels of B-cells are elevated in the third trimester for pregnancies complicated by FGR (Bartha & Comino-Delgado, 1999; Xiong et al. 2012). The higher placental B-cell infiltration is unlikely to result from a fetal systemic elevation since B-cell number in the umbilical cord is lower with FGR (Xiong et al. 2012). Therefore, specific recruitment or retention of B-cells in the placenta appears to occur in pregnancies complicated by FGR. As with T-cells, maternal B-cells are retained in the intervillous space of the placenta, potentially drawn in by the chemokine CCL20 (Solders et al. 2019b). Generally, CCL20 levels are increased by inflammatory signalling pathways (Zhao et al. 2014). How placental expression of CCL20 is regulated, and whether elevated levels of this chemokine are involved in the B-cell infiltration of FGR placentas, remains to be determined.

In this study, placental levels of macrophages or neutrophils were not associated with complications during pregnancy. The lack of altered neutrophil levels in these placentas with pregnancy complications was expected. A major role of neutrophils is as primary defence against infections by both bacteria and eukaryotic pathogens (Mayadas et al. 2014). Therefore, our finding suggests that it is unlikely that there is an active infection in these placentas associated with pregnancy complications. This argument is further supported by the lack of bacteria and eukaryotic pathogens in placental villous tissue (Lager et al. 2018; de Goffau et al. 2019). This strengthens the hypothesis that there is a specific recruitment of CD8\(^+\) T-cells into the placenta in pre-eclampsia and reduced fetal growth, not a general leukocyte infiltration. The underlying cause of increased levels of placental T-cells in complicated pregnancies is likely due to an immunological reaction rather than a response to pathogenic infection.

Morphologically, we report that placental vasculature was not affected in pre-eclampsia or FGR. However, placental abnormalities were more frequently detected in complicated pregnancies than in healthy pregnancies. This was observed both in the villous tissue biopsies and in the edge biopsies. Of particular note is that maternal vascular malperfusion was reported more often in pre-eclampsia and SGA pregnancies. This is in accordance with previous observations (Wright et al. 2017; Hendrix et al. 2019). Maternal vascular malperfusion is a placental injury associated with altered intervillous and uterine blood flows, often resulting in hypoxia of the tissue (Ernst, 2018). This further supports placental hypoxia as a possible primary mechanism for the increased leukocyte infiltration into placental tissue observed in this study.

This study has limitations as well as strengths. To assess the presence of B-cells, macrophages, neutrophils and T-cells, we utilized one antibody per cell type. Therefore, we are unable to assess differing subtypes of these cells or distinct cell types not detected by the selected antibodies. For instance, the frequency of infiltration of CD4\(^+\) T-cells in these placentas remains to be determined. Combining multiple antibodies to detect, for example B-cells or macrophages, may provide additional information on how these immune cells are affected by pregnancy complications. Due to the large number of placentas investigated, we limited the immunohistochemical staining to one antibody per leukocyte type. We have not assessed presence of syncytial debris, syncytial knots, or apoptotic nuclei in this cohort of placentas. Changes in frequency of such features in the placenta may be associated with pregnancy complications (Burton & Jones, 2009; Chamley et al. 2011). These features all represent interesting areas to be addressed in future studies.

The current study also has several important strengths. Among these strengths are that the placental samples utilized are from a large cohort of very well-characterized pregnancies, allowing us to carefully match the cases and controls based on several important maternal, fetal

| Table 7. Pathologist scoring comparing term and preterm placenta |
|-------------------|------------------|------------------|
| Class             | Villous tissue – preterm birth | Edge biopsy – preterm birth |
|                   | (95% CI)          | (95% CI)          |
| Abnormal\(^*\)    | 1.33 (0.59–3.01)  | 2.37 (0.90–6.25)  |
|                   | \(P = 0.4960\)    | \(P = 0.0817\)    |
| Calcification     | NA (0.32)         | NA (0.04–2.49)    |
|                   | \(P = 0.2735\)    | \(P = 0.0062\)    |
| Immature          | 3.98 (1.48–10.69) | 20.85 (5.34–81.44) |
|                   | \(P = 0.0062\)    | \(P < 0.0001\)    |
| Maternal          | 0.87 (0.10–7.63)  | 1.34 (0.36–5.07)  |
| vasculature       | \(P = 0.9066\)    | \(P = 0.6628\)    |

Odds ratios of pre-eclampsia and SGA (95% CI) and \(P\)-value for the unadjusted measurements. *All pathological classifications combined (a placenta was classified as abnormal if it displayed signs of one or more of the pathologies listed in Table 3). Analysis: logistic regression. For some diagnoses, too few placentas with the pathology were observed for statistical analysis. Analysis based on \(n = 263\) (villous tissue) or \(n = 166\) (edge biopsy). NA: not defined for villous tissue.
and pregnancy characteristics. We processed the matched samples together in parallel through handling, staining and analysis. This minimized the influence of batch effects on the results and interpretation of relevance for pregnancy outcomes. Another important strength of this study is that it includes a large number of placentas, all of which were analysed while blinded to case or control status, gestational age and maternal characteristics.

Conclusion

In conclusion, lesions and accumulation of specific immune cells in the placential tissue are more common in FGR and pre-eclampsia than in uncomplicated pregnancies.

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**Additional information**

**Data availability statement**

The data that support the findings of this study are available upon request from the corresponding author. Requests will require a formal Data Transfer Agreement: data are not publicly available due to the terms of the ethical approval.

**Competing interests**

None.

**Author contributions**

G.C.S.S., D.S.C.-J., N.J.S. and F.A.J. conceived the experiments; G.C.S.S., D.S.C.-J. and S.L. designed the experiments; S.L. organized the experiments, designed the image analysis applications, and carried out data management; E.C. and L.H. performed the experiments; S.L., E.E., M.L., C.Y. and E.C. analysed the images; N.J.S. and F.A.J. performed the pathologist scoring of images; U.S. matched cases and controls, carried out the statistical analysis; and all authors contributed to the writing of the article. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

**Funding**

This study was supported by grants from the Medical Research Council (UK; MR/K021133/1) and the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre (Women’s Health theme). The funding sources had no involvement in study design, data collection, analysis or interpretation, article preparation or deciding to submit the article for publication.

**Acknowledgements**

The authors are grateful to all participants of the POP study. We are also grateful to Leah Bibby, Josephine Gill, Katrina Holmes, Ryan Millar and Samudra Ranawaka for their technical assistance in biobank management, sample collection and preparation, Keli Phillips for cutting all the sections, Zoe Mitchell and James Warner for staining the sections, and the Histopathology/ISH Core Facility at Cancer Research UK Cambridge Institute for scanning the slides.

We tested the hypothesis that placental histopathological findings differ comparing complicated pregnancies with healthy pregnancies. Placental samples were collected after delivery from a large prospective cohort study of women in their first pregnancy. Placental samples were stored for histology and,
following completion of the study, samples from women that developed pre-eclampsia or gave birth to a growth restricted infant were compared to matched healthy controls. In this study, we show that placentas from pregnancies complicated by pre-eclampsia or fetal growth restriction more often had pathological findings, as assessed by two experienced placental pathologists, blinded to case or control status. Quantitative immunohistochemistry, also performed blind to case control status, was also employed, and demonstrated that samples from cases contained more immune cells. At present, an incomplete understanding exists of the underlying causes for fetal growth restriction and pre-eclampsia. But here we show objective evidence for associations with placental pathology and immune cell infiltration, which cannot be explained by ascertainment bias.

Keywords
endothelial cells, fibrin, image analysis, leukocytes, pathologist scoring, pregnancy complication, small-for-gestational age, stereology, villitis of unknown aetiology, villous tissue

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document.