# Additional file 1

## Title: Immune Changes in Pregnancy: Associations with Pre-existing Conditions and Obstetrical Complications at the 20th Gestational Week - A Prospective Cohort Study

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**Methods S1:** Additional information on data preprocessing and modeling

*Meso Scale Diagnostics data normalization*

We measured 47 inflammatory markers using the V-PLEX Human Biomarker 54-Plex Kit from Meso Scale Diagnostics (MSD). Due to low-quality assay validation, we excluded all measurements from the Th17 panel, resulting in 47 assays on six panels to be included in the analysis.

To address the batch effect exerted by the individual 96-well plate setup, we explored the corrective effects of three data pre-processing methods and four normalization methods with the aim of removing batch effects at panel level.

The MSD V-PLEX kit is based on electrochemiluminescence technology, where light emission from SULFO-TAG labels is measured as light intensity ("signal"). The signal values are within the dynamic range of the assay linearly associated with the concentration of the measured target. While this principle is used for concentration determination by a measured standard curve, we here used the signals directly to avoid any noise introduced by the measurement of a standard curve.

Due to the multiplex setup, plate-based batch effects were assessed by panel, as technical variation was assumed to be equal across the four to ten assays measured per panel. Our first aim was to remove any observed variation based on the 16 plates the samples were run on. We compared the combination of three pre-processing methods: *a*) log2-transformation, *b*) method *a* plus removal of outliers based on principal component analysis (PCA)[1], and *c*) method *b* plus ComBat batch correction[2], and four normalization methods: *i*) no normalization, *ii*) median normalization[3], *iii*) quantile normalization[4], and *iv)* MA normalization[5]. To assess the effectiveness of normalization, we performed a visual inspection of PCA plots, density plots, and box plots.

Based on visual inspection of the PCA plots of the log2-transformed data, we defined individual outlier limits for the six panels removing samples that exceeded these limits. Consequently, we removed 17 outliers on Angio1, 13 outliers on Chem1, 11 outliers on Cyto1, 41 outliers on Cyto2, 8 outliers on Pro1, and 9 outliers on Vascu2 (see limits on Additional file 1: Fig. S3).

We found median normalization to sufficiently remove all batch effects observed at PC1 and PC2 and yielded overlapping curves with normal or near-normal distributions for the individual assays observed on density and box plots. Similar results were found for MA normalization, while the other methods yielded varying poorer results.

*Similarity reduction of markers using Hobohm II*

Hierarchical clustering showed high similarity between markers, indicating redundancy in the data set. To address this, we employed the Hobolm II algorithm as outlined in Hobohm *et al*, 1992[6]. We used Spearman correlation as the correlation metric and tested cut-off levels at 0.3, 0.4, 0.5, 0.6, 0.7, 0.8,

0.9, and 1 (Additional file 1: Fig. S1). We chose a cut-off of 0.5, resulting in 41 markers. Using this cut-off, we did not observe issues of collinearity in any of the statistical models.

*Bayesian regression models*

For all statistical analyses we employed Bayesian regression models. All models were fit using rstanarm or brms[7–9]. Unless otherwise specified, all models were run for 10,000 iterations (5,000 warm-up and 5,000 sampling) with default settings for the sampler. Convergence was assessed by calculating r-hat statistics, looking for divergences and making sure the sample did not exceed the maximum tree depth after warm-up. A model had converged if and only if (1) all r-hat values < 1.01, (2) no divergences, and (3) no iterations exceeding the maximum tree depth. All parameters were centered prior to model fitting and MSD inflammatory markers were standardized so that the interpretation follows changes in standard deviations. Furthermore, we also inspected correlations of the posterior distributions to identify issues of collinearity.

*Bayesian robust linear regression*

The Bayesian robust linear regression extends the classical ordinary least squares by assuming a Student t-distribution, thereby accommodating outliers. The degree of freedom is estimated directly from the data. Formally,

$$y \sim t(\nu,\ \mu,\ \sigma)$$

in which μ is the sum over the intercept and covariates,

$$\mu\ = b_0 + \sum_k \beta_k x_k$$

To complete the model, we specify a set of priors across the parameters in the model,

$$\beta_0 \sim N(0,\ 2.5)$$

$$\beta_k \sim t(7,\ 0,\ 0.5)$$

$$\sigma \sim N(0,\ 1)$$

$$\nu \sim N(0,\ 1)$$

Posterior model checking was done by simulating 100 draws from the posterior and comparing with the observed distribution.

*Cause-specific parametric proportional hazards models*

For analyzing the duration, or time to failure, we employed cause-specific parametric proportional hazards survival models, as implemented in rstanarm[8]. This takes into account that some women were lost to follow-up and are thus censored, and that there may be competing outcomes (e.g., induced labor, acute cesarean section, or scheduled cesarean section is a competing outcome to

spontaneous vaginal birth). We compared two baseline hazards (cubic B-spline and M-spline) for each outcome and visually inspected the estimated baseline hazard curve, versus the observed curve. Model priors follow default settings, except the regression coefficients ($\beta_k$) which were assigned a more conservative prior,

$$\beta_k \sim t(7,\ 0,\ 0.5)$$

The prior induces regularization by forcing coefficients towards zero, i.e., a null effect.

*Birthweight to gestational age ratio*

For the birth weight to gestational age ratio, the values were standardized according to the mean and standard deviation estimated from the Danish Medical Birth Registry (DMBR), including all births in 2020 (Additional file 1: Table S6). From the posterior, we defined a transformation,

$$P(X \leq x) = F(X|\mu, \sigma, \nu) - 0.5$$

in which X is the estimated value of the marker, and F is the Student-t cumulative density distribution parametrized by the mean, standard deviation, and degrees of freedom estimated from the DMBR. The parameters were estimated using a Bayesian robust linear regression. The resulting value represents the change from the 50th percentile for a one-unit change in the variable.

## References

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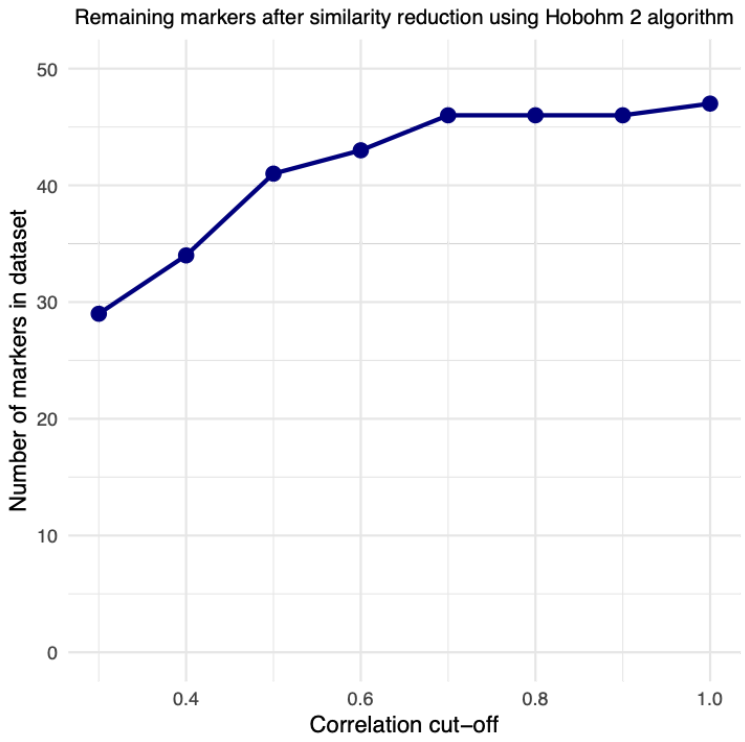


**Fig. S1**: Number of markers remaining after pruning by the Hobohm II algorithm according to eight cut-off levels for the Spearman correlation coefficient.

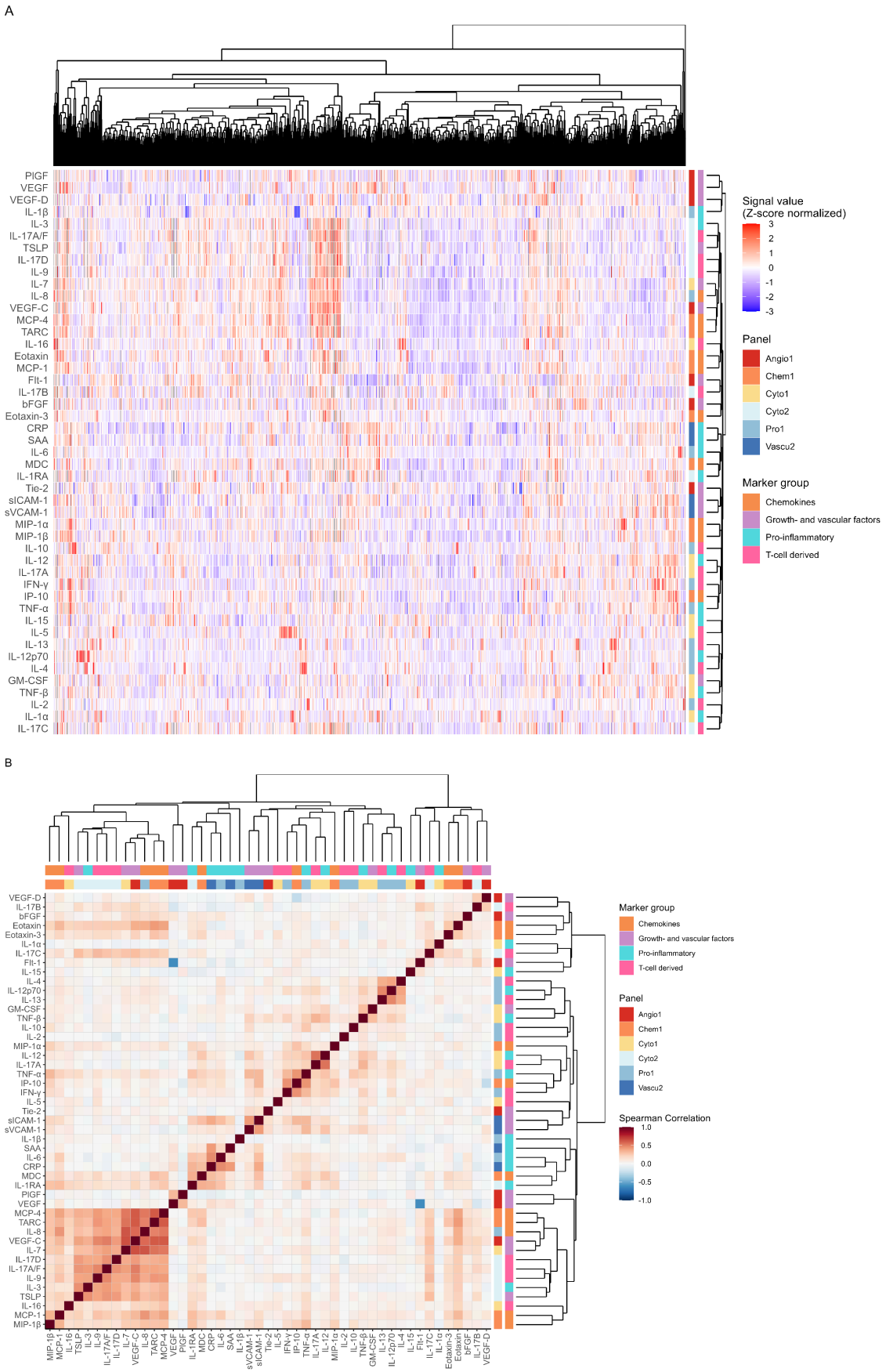


**Fig. S2**: Correlation between MSD markers. (a) Heatmap of women x markers, (b) Heatmap of markers x markers.

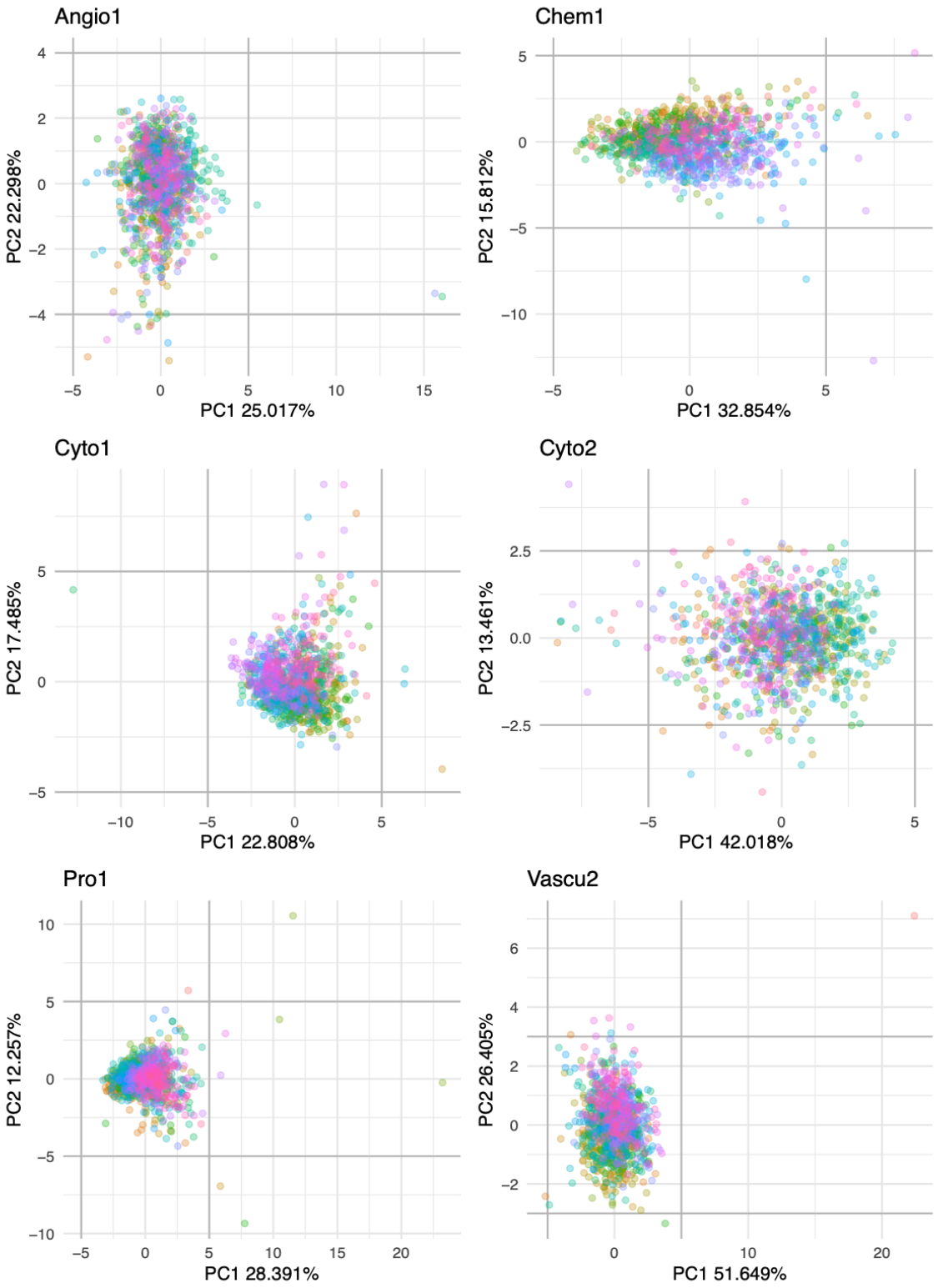


**Fig. S3**: PCA plots for log2-transformed signal values colored by plate for the six MSD panels. Grey lines indicate selected limits for outlier detection.

**Table S1**: Outcome descriptions, definitions, adjustments, and exclusion criteria.

| Outcome | Definition | Outcome-specific adjustments | Exclusion criteria |
|---|---|---|---|
| Pre-eclampsia | Blood Pressure > 140 mmhg systolic and/or 90 mmHg diastolic & proteinuria | Previous number of live births,<br>Smoking,<br>PAPP-A MoM,<br>βHCG MoM,<br>ART (divided into IUI or IVF),<br>Ethnicity | Pre-gestational hypertension<br>Gestational hypertension diagnosed before inclusion<br>pre-eclampsia in previous pregnancy |
| Spontaneous vaginal birth | Birth not assisted by cesarean section or induction | Smoking,<br>Hemorrhage in early pregnancy,<br>UTI in current pregnancy,<br>Conisatio | n.a. |
| Gestational age at birth | Gestational age calculated from ultrasound. | Smoking,<br>Hemorrhage in early pregnancy,<br>UTI in current pregnancy,<br>Conisatio | n.a. |
| Preterm birth | Gestational age < 37+0 | Smoking,<br>Hemorrhage in early pregnancy,<br>UTI in current pregnancy,<br>Conisatio | |
| Gestational diabetes mellitus (GDM) | Based on a two-hour 75 gram oral glucose tolerance test (OGTT); GDM with glucose ≥ 9.0 mmol/l in capillary whole blood or venous plasma | PCOS,<br>GDM in prior pregnancy | GDM diagnosed before inclusion<br>Diabetes Mellitus |
| Birth weight / gestational age ratio | Birth weight recorded immediately after birth by midwife or doctor. | | |
| Severe Postpartum hemorrhage | 1000 mL blood loss or more within 24 hours | ART (divided into IUI or IVF),<br>Hemorrhage in early pregnancy,<br>Cesarean section in prior pregnancy | |
| Acute cesarean section | Delivery by acute cesarean section, | Cesarean section in prior pregnancy | |

| | | | |
|---|---|---|---|
| | any degree | | |
| Any complication | Pre-eclampsia, GDM, Severe PPH, Preterm birth, or acute cesarean section. | Previous number of live births,<br>Smoking,<br>PAPP-A MoM,<br>βHCG MoM,<br>ART (divided into IUI or IVF),<br>PE in prior pregnancy,<br>GDM in prior pregnancy,<br>PCOS,<br>UTI in current pregnancy,<br>Cesarean section in prior pregnancy,<br>Conisatio,<br>Ethnicity | Pre-gestational hypertension<br>Gestational hypertension diagnosed before inclusion<br>pre-eclampsia in previous pregnancy<br>GDM diagnosed before inclusion<br>Diabetes Mellitus (type 1 or 2) |

Abbreviations: ART, assisted reproductive technology; GDM, gestational diabetes mellitus; IUI, intrauterine insemination; IVF, in vitro fertilization; MoM, multiple of the median; n.a., not applicable; OGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; PE, Pre-eclampsia; PPH, postpartum hemorrhage; UTI, urinary tract infection

**Table S2**: Hyperparameter values

| **Model** | **Definition** | **Outcome-specific adjustments** |
|---|---|---|
| Logistic regression | L1 penalty | 1e-8 … 1 (log spaced) |
| LightGBM | Number of estimator | 1 … 200 |
| | Learning rate | 0.0001 … 1 (log spaced) |
| | Max depth | 3 … 30 |
| | Subsample, individuals | 0.2 … 0.8 |
| | Subsample, feature | 0.2 … 0.8 |
| | Mininum child samples | 50 … 200 |
| | Maximum tree leaves | 6 … 50 |
| | L1 penalty | [0, 0.01, 1, 2, 5, 7, 10, 50, 100] |
| | L2 penalty | [0, 0.01, 1, 2, 5, 7, 10, 50, 100] |

**Table S3**: Comparison of PREGCO and the Danish Medical Birth Registry (DMBR).

| **Variable** | | **PREGCO (n=1,049)** | **DMBR 2020 (n=60,573)** | **Difference** |
|---|---|---|---|---|
| Maternal age, years* | | 31.7 (4.5) | 30.7 (4.7) | 1.0 (0.72; 1.27, p < 0.001) |
| Pre-pregnancy BMI, $m/kg^2$* | | 24.1 (4.8) | 24.8 (5.4) | -0.7 (-0.99; -0.41, p < 0.001) |
| Smoking during pregnancy** | | 40 (3.8%) | 4,660 (7.7%) | 0.49 (0.35; 0.67, p < 0.001) |
| Parity** | 0 | 576 (54.9%) | 22,644 (37.3%) | p < 0.001 |
| | 1 | 375 (35.7%) | 20,421 (33.7%) | |
| | 2 | 81 (7.7%) | 10,011 (16.5%) | |
| | 3+ | 17 (1.6%) | 7,507 (12.4%) | |
| Number of Pregnancy Losses** | 0 | 810 (77.2%) | 52,145 (86%) | p < 0.001 |
| | 1 | 194 (18.5%) | 6,965 (11.5%) | |
| | 2 | 29 (2.8%) | 1,230 (2.0%) | |
| | 3+ | 16 (1.5%) | 282 (0.5%) | |
| Sex of child, male** | | 521 (50.5%) | 30,983 (51%) | 0.96 (0.86; 1.07, p=0.44) |

* Z-test; **$\chi^2$ test

**Table S4**: Variables and sources

| Group | Name | Timepoint measured / recorded and Source |
|---|---|---|
| Maternal Characteristics | Maternal age | Pre-pregnancy, EHR |
| | BMI | Pre-pregnancy, EHR |
| | Smoking | At inclusion, EHR |
| | Ethnicity | Pre-pregnancy, EHR |
| Current Pregnancy | ART | Pre-pregnancy, EHR |
| | Gestational age at inclusion, based on ultrasound | At inclusion, EHR |
| | GDM in current pregnancy | At inclusion, EHR |
| | COVID-19 antibodies | 8-12th week scan and at inclusion, EHR |
| | Early hemorrhage | At inclusion, EHR |
| Fetal characteristics | Sex of child | At inclusion, EHR |
| | PAPP-A, MoM | 8-12th week scan, EHR |
| | βHCG, MoM | 8-12th week scan, EHR |
| | Gestational age, based on ultrasound | 8-12th week scan, EHR |
| | Abdominal circumference | At inclusion, EHR |
| | Head circumference | At inclusion, EHR |
| | Femur length | At inclusion, EHR |
| Prior pregnancies | GDM in prior pregnancy | At inclusion, Questionnaire |
| | Pre-eclampsia in prior pregnancy | At inclusion, Questionnaire |
| | PPH in prior pregnancy | At inclusion, Questionnaire |
| | Preterm labor in prior pregnancy | At inclusion, Questionnaire |
| | Previous number of cesarean sections | At inclusion, Questionnaire |
| | Previous number of live births | At inclusion, Questionnaire |
| | Previous number of pregnancy losses | At inclusion, Questionnaire |
| Prior diseases | Conisatio | At inclusion, Questionnaire |
| | Endometriosis | At inclusion, Questionnaire |
| | PCOS | At inclusion, Questionnaire |

Abbreviations: ART, assisted reproductive technology; BMI, body mass index; EHR, Electronic Health record; GDM, gestational diabetes mellitus; MoM, multiples of medians; PCOS, polycystic ovary syndrome

**Table S5**: Results from cross-validation of machine learning models. The highest ranking model for each outcome is highlighted in bold, based on the binary cross entropy from the inner CV.

| | | Development (inner CV) | | | Interval validation (outer CV) | |
|---|---|---|---|---|---|---|
| **Outcome** | **Model** | Binary Cross Entropy | ROC-AUC | AUPRC | ROC-AUC | AUPRC |
| Acute Sectio | **LASSO** | **0.216** | **0.638** | **0.130** | **0.590 (0.515-0.667)** | **0.086 (0.057-0.126)** |
| | LightGBM | 0.216 | | | | |
| Any complication | **LASSO** | **0.599** | **0.630** | **0.457** | **0.615 (0.576-0.655)** | **0.421 (0.367-0.477)** |
| | LightGBM | 0.602 | | | | |
| Gestational Diabetes | LASSO | 0.268 | | | | |
| | **LightGBM** | **0.266** | **0.697** | **0.219** | **0.708 (0.644-0.766)** | **0.176 (0.126-0.238)** |
| Pre-eclampsia | LASSO | 0.150 | | | | |
| | **LightGBM** | **0.149** | **0.655** | **0.125** | **0.672 (0.580-0.758)** | **0.073 (0.042-0.115)** |
| Preterm birth | LASSO | 0.172 | | | | |
| | **LightGBM** | **0.171** | **0.610** | **0.118** | **0.564 (0.460-0.66)** | **0.071 (0.041-0.132)** |
| Severe PPH | LASSO | 0.349 | | | | |
| | **LightGBM** | **0.349** | **0.628** | **0.198** | **0.587 (0.530-0.638)** | **0.152 (0.117-0.193)** |

**Table S6**: Birth weight-gestational duration ratio parameters estimated from the Danish Medical Birth Registry (DMBR).

| Parameter | Median | 95% bCI |
|---|---|---|
| μ | 12.61 | 12.59-12.62 |
| σ | 1.54 | 1.53-1.56 |
| ν | 6.88 | 6.54-7.24 |