Neonatal inflammation, stress and growth factors after vaginal delivery, pre-labour, and in-labour caesarean section: a retrospective cohort study

Pia Kiilerich[1], Rikke Cortes[1], Ulrik LAUSTEN-THOMSEN [2], Nis BORBYE-LORENZEN[1], and Kristin Skogstrand[1]

[1]Statens Serum Institut
[2]Rigshospitalet

June 9, 2020

Abstract

Objective To explore the effect of different delivery modes, vaginal delivery compared to caesarean section with or without initiation of labour, on the immune system and brain of the infants. Further, we aimed to elucidate gender and gestational ages' effect on these biomarkers. Design Retrospective case control study Setting Dried blood spots from new-born screening biobank drawn 2-4 days after birth Population or Sample Mature new-borns divided into delivery by pre-labour (n=714), in-labour caesarean section (n=655), and vaginally (n=5897). Method The samples were analysed for inflammatory markers (IL-18, MCP-1, CRP, sTNF RI), stress- (HSP-70), growth- (EGF, VEGF-A), and neurotrophic factors (BDNF, NT-3, S100B). Main Outcome Measures Delivery by caesarean section with or without initiation of labour Results The neonatal levels of inflammatory and stress-markers were significantly lower, while the levels of growth factors were higher after pre-labour caesarean section compared to vaginal delivery. The biomarker levels were similar after in-labour caesarean section and vaginal delivery. Males had generally higher levels of inflammation and lower levels of growth and neurotrophic factors. Overall, the levels of inflammatory markers increased, and the growth factors decreased with increasing gestational age. Conclusion The biomarker levels indicates that the labour process has an important effect on the foetal immune system and level of stress, regardless if the delivery ends with caesarean section or vaginal birth. Funding "Læge Sofus Carl Emil Friis og hustru Olga Doris Friis' legat" and "Fonden til Lægevidenskabens Fremme". Keywords Inflammation; brain; CODIBINE; caesarean section; dried blood spot samples

Objective

To explore the effect of different delivery modes, vaginal delivery compared to caesarean section with or without initiation of labour, on the immune system and brain of the infants. Further, we aimed to elucidate gender and gestational ages' effect on these biomarkers.

Design

Retrospective case control study

Setting

Dried blood spots from new-born screening biobank drawn 2-4 days after birth

Population or Sample

Mature new-borns divided into delivery by pre-labour (n=714), in-labour caesarean section (n=655), and vaginally (n=5897).

Method

The samples were analysed for inflammatory markers (IL-18, MCP-1, CRP, sTNF RI), stress- (HSP-70), growth- (EGF, VEGF-A), and neurotrophic factors (BDNF, NT-3, S100B).

Main Outcome Measures

Delivery by caesarean section with or without initiation of labour

Results

The neonatal levels of inflammatory and stress-markers were significantly lower, while the levels of growth factors were higher after pre-labour caesarean section compared to vaginal delivery. The biomarker levels were similar after in-labour caesarean section and vaginal delivery. Males had generally higher levels of inflammation and lower levels of growth and neurotrophic factors. Overall, the levels of inflammatory markers increased, and the growth factors decreased with increasing gestational age.

Conclusion

The biomarker levels indicates that the labour process has an important effect on the foetal immune system and level of stress, regardless if the delivery ends with caesarean section or vaginal birth.

Funding

"Læge Sofus Carl Emil Friis og hustru Olga Doris Friis' legat" and "Fonden til Lægevidenskabens Fremme".

Keywords

Inflammation; brain; CODIBINE; caesarean section; dried blood spot samples

Tweetable abstract: Pre-labour C-section leads to altered biomarkers in new-borns compared to in-labour C-section or vaginal delivery

Introduction

Caesarean section (CS) is a well-known and potentially life-saving surgical procedure. The global rate of deliveries by CS has increased from 12.1% in 2000 to 21.1% of deliveries in 2015 (1). It is estimated that 9-19% of CSs can be justified by medical indications (2, 3), and the World Health Organization currently recommends CS for up to 15% of deliveries(4). Yet, national frequencies varies greatly and range from 0.6% in South Sudan (1) to 55-65% in Brazil (5).

Recently, increasing evidence has shown that birth delivery mode has an impact on child health (6). CS is associated to early complications such as birth asphyxia, respiratory disturbances (7), soft tissue injury (8), and to neurological and psychological complications such as autism spectrum disorders (9), ADHD (10), psychosis (11), anxiety, depression, and sleep disturbances (12). Notable CS-associated late-term complications for the child includes implications of the immune system: systemic connective tissue disorders, juvenile arthritis (13), inflammatory bowel disease, immune deficiencies, asthma (14, 15), sepsis (16), type 1 diabetes (14), celiac disease (17), and autoimmune diseases (12).

Compared to infants delivered vaginally, a delivery by CS is thought to alter the short-term immune response in new-borns by variation of bacterial colonization of the intestinal tract, due to lack of exposure to the vaginal and anal microbiota during delivery (18, 19). Further, the level of foetal stress during CS is lower compared to vaginal delivery (VD), as both the initiation of birth and the contractions during VD may trigger many biological effects (20). Pre-labour CS, i.e. CS before the onset of labour, are more often associated with several of the above mentioned complications compared with in-labour CS (7, 11, 13, 14, 17), suggesting that important endocrine, physiological and biochemical processes in the infant are initiated by labour. Most biomarker studies reported regarding CS versus VD have excluded cases with in-labour CS, thus the studies cannot explain if the differences found are due to the surgery or the lack of labour.

Infant males are known to be more fragile due to yet unknown biological mechanisms (21); they are more often prematurely spontaneously aborted (22), born premature or late, stillborn, have a higher infant mortality, and are also more often delivered by CS (23).

Accordingly, we aimed to explore the delivery mode's effects on the immune system and brain per se of the infants, by measuring inflammatory, stress, growth, and neurotrophic biomarkers in more than 7000 neonatal dried blood spot samples (DBSS). Further, we wanted to elucidate potential gender- and gestational age-dependent differences of the biomarker levels.

Materials and Methods

Sample selection

The CODIBINE cohort comprises all new-borns born with gestational age (GA)<37 weeks (n=7946) in Denmark between March 2009-March 2011, and maturely born controls with GA[?]37 weeks (n=7946) matched by birth hospital and day. The blood samples were drawn from a heel prick as DBSS for routine new-born screening. Samples are shipped by mail and stored at the Danish New-born Screening Biobank, Statens Serum Institut, at -24degC after screening (24). Our cohort is linked with data from the Danish Medical Birth Register (MBR) (25) using the Danish Civil Registration System (26). This is the first of a series of papers from the project called CODIBINE, Correlations and Diagnoses for Biomarkers in New-borns, aiming to explore biomarkers in new-borns correlated to birth, complications and diagnoses later in life.

In the present study, we reduced the cohort to include mature births, with samples drawn 2-4 days after birth, in total 7266 individuals: 714 delivered by pre-labour CS, i.e. CS before onset of labour, 655 by in-labour CS, i.e. CS after initiation of labour, and 5897. Table 1 shows the demographic data for the mothers and new-borns.

Exclusion criteria were: active dissent to participation in scientific studies (as registered in the Danish national register for use of biological tissue, "Vaevsanvendelsesregisteret"), lack of or insufficient sample material, incomplete civil registration number, and non-participation in the National New-born Screening Program.

The study was supported by "Laege Sofus Carl Emil Friis og hustru Olga Doris Friis' legat" and "Fonden til Laegevidenskabens Fremme". The warded grant did not include external peer review for scientific quality or priority assessment. The funders were not involved in conducting research and writing the paper.

Sample analysis

The samples were analysed using an in-house developed multiplex assay based on Meso-Scale Discovery (MSD) technolog, targeting 3 biomarkers for inflammation (Interleukin-18 (IL-18), Monocyte Chemotactic Protein (MCP-1), C-Reactive Protein (CRP)), 1 anti-inflammatory marker (soluble Tumor Necrosis Factor 1 (sTNF RI)), 1 biomarker for stress (Heat Shock Protein-70 (HSP70)), 2 growth factors (Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor A (VEGF-A)), and 3 neurotrophic biomarkers for brain development and/or damage (Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and S100 calcium binding protein B (S100B)).

All antibodies were purchased from RnDsystems, with the exception of anti-S100B, which was purchased from Sigma-Aldrich (capture) and DAKO (detection), respectively. The capture antibodies were purchased biotinylated, except for anti-S100B that was in-house purified using protein A column, and biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher) as per manufacturers instructions. The biotinylated antibodies were diluted to the concentration 0.1µg/ml for CRP and 10µg/ml for the other antibodies, bound to different linkers 1-10 (MSD) according to manufacturer's instructions, and finally added to each plate well (50µl/well, U-plex plates, MSD), and incubated at room temperature (RT) for 1 hour. After washing with washing buffer (PBS containing 0.05% Tween 20), the plates were stored at 4°C until use. Calibrators

and controls were prepared by recombinant antigens diluted in diluent 7 (MSD). All detection antibodies were sulfo-tagged using MSD Gold Sulfo-tag NHS-Ester (MSD) as per manufacturers instructions, and were mixed together to a final concentration of 0.1µg/ml of each antibody.

The DBSS were punched as 2x3.2mm disks in microtiter wells using DBS Puncher® instrument (Perkin Elmer), added 130 µl extraction buffer (PBS containing proteaseinhibitor, as earlier described (27)) to each well, sealed and extracted for 1 hour at RT on a shaker set at 450 rpm. After extraction, 50µl extract was aspirated from each well and transferred to the pre-coated plates with a pipetting robot (Biomek 4000, Ramcon) together with calibrators and controls. The plates were sealed and incubated on a shaker set at 600 rpm for 2 hours at RT. After washing, detection antibodies were added to each well followed by 2 hours incubation at 600 rpm at RT.

Finally, the plates were washed, added 2xRead buffer T (MSD), and immediately read at the QuickPlex reader (MSD).

Concentrations of the biomarkers were calculated from the calibration curves with 4 parameter logistic fit using Discovery Workbench 4.0 software (MSD).

Analytical characteristics are shown in table 2. Values measured below or above the working ranges were set to the lowest or highest measurable concentration.

Statistics

For each of the ten biomarkers the data was log-transformed using the natural logarithm in order to obtain normal distribution. The mode of delivery was stratified into three groups; VD, pre-labour CS and in-labour CS, as classified by obstetrician at the time of birth. Two-way ANOVA was used to test for overall difference between birth type, where GA was included in the model as well as the interaction, and to test for the overall difference between genders, where birth type was included in the model as well as the interaction. A t-test with un-pooled variance was used to detect pairwise significant differences with Holms method (28) to adjust for multiple testing.

One-way ANOVA was used to test for the overall difference of the biomarkers between gestational age. A pairwise t-test with un-pooled variance, adjusted for multiple testing using Holms method, was used to test for differences between the weeks.

Maternal age, BMI, and neonatal birth weight and age at sampling were initially included in the models, but did not contribute to the explanation of the variation, and these parameters were therefore excluded from the final models (data not shown). The birth weight and GA was lower in the pre-labour CS group. Birth weight was included in the first calculations, but did not make any differences as long as GA was included, and thus were excluded in the final calculation.

No difference was found in the use of anaesthetics between pre-labour and in-labour CS (data not shown), thus we did not correct for this in the analysis. The analysis was re-run excluding the cases with pre-labour rupture of the membranes (PROM). No difference in the result was found (data not shown). Only 0.8% of the infants were registered with an infection the first 4 days after birth, thus this was not adjusted for in the statistics.

All statistical analyses and figures were performed using the dplyr (29) and ggplot2 (30) packages in R software version 3.5.2.

Results

Infants born by pre-labour CS had significantly decreased levels of the inflammatory markers (CRP, MCP-1, IL-18) and the stress-marker HSP70, and significantly increased levels of the growth factors (VEGF and EGF) compared to infants born by VD. Comparing pre-labour CS with in-labour CS showed a similar

pattern: CRP, MCP-1 and HSP70 were significantly decreased, and VEGF was increased, in infants born by pre-labour CS compared to infants born by in-labour CS. When comparing in-labour CS to VD, only CRP was significantly different. We found no delivery form depending differences in neonatal levels of the neurotrophic factors (S100B, BDNF and NT-3), or the anti-inflammatory marker sTNF RI (Figure 1).

Most biomarkers were different between genders: males had significantly lower levels than females of the anti-inflammatory marker sTNF RI, the growth factors (EGF and VEGF), and the neurotrophic factor BDNF. The inflammatory markers CRP and MCP-1 were higher in males compared to females. Delivery form has an overall effect on S100B and IL-18 in males, but not in females. The gender differences were generally more significant after VD (CRP, MCP-1, sTNF RI, EGF, BDNF, VEGF), than after in-labour (CRP, BDNF) and pre-labour CS (CRP, VEGF) (Figure 2).

We saw an overall increase of the inflammatory markers CRP, IL-18, and MCP-1 from GA 37-42, and a decrease of the anti-inflammatory marker sTNF RI. The stress-marker HSP70 decreased from GA 38-40. The growth factors EGF and VEGF, and the neurotrophic factor S100B, decreased overall in samples from GA 37 to 42, while no overall significant difference was found for the neurotrophic factors BDNF and NT-3 (Figure 3).

There was a significant difference in infants' birth weight between delivery forms, where the heaviest infants are born by in-labour CS and the lightest by pre-labour CS (table 1). Birth weight was included in the first calculations, but did not make any differences as long as GA was included in the final model, and thus were excluded in the final calculation. The maternal BMI was higher for mothers in the CS group (no matter type) compared to VD, and the maternal age was higher in the pre-labour CS group compared to the other groups. Neither of the factors could explain any of the differences found in the biomarkers. The age at sampling could not explain the differences observed in biomarkers between the different delivery forms, neither could it explain the differences in males and females. There were significantly more cases of PROM before in-labour CS and VD. Removing all cases with PROM did not change the results.

Discussion

Main findings:

In this study, the neonatal levels of inflammatory and stress-markers were generally lower, and the levels of growth factors were higher after pre-labour CS compared to after VD or in-labour CS. The differences in biomarkers could not be explained by the higher incidence of PROM before in-labour CS and VD. Accordingly, the data suggests that the labour process has an important physiological effect on the foetal immune system and level of stress, regardless if the delivery ends with caesarean section or vaginal birth.

Strengths and limitations:

The strengths of this study are the study size, and the unbiased and random selection of the participants from all of Denmark, meaning that there was no selection for social status, income, race etc. All samples were handled in the same way, and analyzed in the same labouratory, thus minimizing analytic variance. We were not able to account for lifestyle factors associated with maternal request for CS or factors increasing the risk of CS. Further, we did not have any information regarding length of labour.

Interpretation

Decreased levels of inflammatory markers in cord blood and neonatal serum after CS compared with VD has been described before (31, 32), but most previous studies did not stratify for the type of CS (pre-labour or in-labour). A few studies regarding CRP have been described, that found similar differences in new-born levels depending on birth form as we describe (33, 34). In a study among teenagers, whose mothers had entered the active phase of labour before CS, spontaneous and toll-like receptor-stimulated cytokine release was increased, compared to controls born by pre-labour CS (35). This concurs with another study showing

that the risk for early childhood infections are higher in children born by pre-labour CS compared to children born by in-labour CS or VD (1, 36). One of the common explanations for the immunological differences after CS and VD has been microbial transmission from mother to child, either by transmission during vaginal birth or by microbial invasion of the amniotic cavity after PROM (32, 36). However, in our study, the exclusion of all cases with PROM did not make any difference in the significance of the biomarker levels. Following national guidelines, prophylactic antibiotics are given during or after both pre- and in-labour CS in Denmark (37), and the hospital stay afterwards are the same (in contrast to after VD where the woman and infant often leaves the hospital a few hours after birth). The explanation for the observed differences in our study is thus more likely to be the influences of stress hormones and/or the physical pressure from the labour process. During VD, cortisol and other stress hormones increase. Elevated cortisol at birth is a known indicator of hypothalamic-pituitary-adrenal axis activation, which is important for regulation of stress and many other body processes (38). The observed sustained inflammatory response 2-4 days after birth in our study after in-labour CS or VD may thus be protective for the foetus later in life, and could possible even explain some of the increased risk for autoimmune and inflammatory disorders after pre-labour CS.

The intracellular inducible HSP70 are one of the major HSPs involved in numerous cellular functions, such as cytoprotection, anti-apoptosis, and immune regulatory effects (39). Increased temperature, exposure to oxidative stress, such as hypoxia, viral infection, and ischemia-reperfusion injury, can induce the expression (39). The fact that HSP70 had similar infant levels after in-labour CS and VD may indicate that the stress effect is the same provided the labour have been initiated, regardless if the delivery ends with CS or VD.

Animal studies have shown that mouse brains have increased contents of norepinephrine, dopamine, serotonin and metabolites of dopamine and serotonin after vaginal deliveries compared to mice delivered by CS. The turnover ratios of the neurotransmitters were also higher in the mouse brains after vaginal delivery, and the later adult mice showed different behavioural patterns (40). We have recently found significantly lower neonatal levels of BDNF in new-borns later diagnosed with autism spectrum disorders (41). In the current study, we did not see any correlation with the neurotrophic markers BDNF, NT-3, and S100B, and the delivery form. This might indicate that neurodevelopment are not dependent on delivery form.

VEGF is a growth factor that stimulates vasculogenesis and angiogenesis after stress, and it is an essential factor for placental development (42). VEGF is expressed at sites of injury and inhibits the activity of nitric oxide synthase, preventing inflammation (43), and it is present at high levels in the central nervous system (44). VEGF is a potent stimulator of angiogenesis in asthma (45), and both VEGF and EGF are stimulators of mucins in the respiratory tract, which concentrations have been reported as positively correlated with asthma disease severity (46, 47). We are not aware of any other studies regarding delivery forms and growth factors. The increased levels after pre-labour CS could indicate that the mechanisms for the increased risk for asthma have been initiated already 2-4 days after birth, but this needs to be more thoroughly investigated in further studies using asthma as an outcome.

Higher concentrations of CRP in neonatal males compared to females has been described before (48). This may be due to hormonal differences, as estradiol (which is highest in females) decreases the production of CRP (49, 50), and increases the gene expression of BDNF (51). Although these early-life differences may simply be an epiphenomenon, it might be the precursors to later in life higher frequencies of different disorders in males, but this needs further investigation. Circumcision is rarely performed in Denmark (52), thus this cannot explain any of the gender biomarker differences (52).

The overall increasing levels of inflammation biomarkers from GA 37-42 concurs with a study showing that the leucocyte count increases with GA up to week 40 (53). The levels of HSP70 decreases until week 40, but then increases in week 41 and 42. This may support the ongoing discussion about earlier deliveries of foetuses after GA week 40 due to increased risk for the overdue foetus (54). The growth factors and S100B decreased for each week of GA, while the neurotrophic factors did not depend on GA. Low GA at birth is a well-known risk factor for later cognitive impairment (55). This effect appears to persist even when born at term, and a study has reported that there are significantly more children receiving special education the

lower the gestational age they were born, even up to GA week 39 compared to week 40 (56). We did not see any differences in the neurotrophic markers that could explain this, but the higher levels of growth factors and S100B could indicate a less mature body and brain.

Conclusion

The results indicate that the labour process is an important and necessary part of delivery for the infants' immune system, and also add to the growing body of evidence suggesting an unnecessary "overproduction" of CS leading to not only maternal and socioeconomic, but certainly also neonatal consequences. The long-term outcome, after pre-labour and in-labour CS, on the developing immune system in the children in this study, remains to be investigated. When the children in the CODIBINE cohort gets older, and thus have obtained an age where more diagnoses have been set, we plan to make a full study on several disorders and birth complications.

Acknowledgements

Thanks to Malene Billsten Zent, Lisette Jensen, Karin Skaarup, and Karina Liebmann Madsen for skilful laboratory analysis.

Disclosure of interests

The authors have no conflicts of interests.

Author contributions

PK: analysing and writing up the work

RC: analysing and writing up the work

ULT: analysing and writing up the work

NBL: analysing and writing up the work

KS: Conception, planning, carrying out, analysing and writing up the work

Details of Ethics Approval

The study was approved by the Danish Ethical Committee (VEK), Project-ID H-6-2014-078 and H-6-2014-079.

Funding

The study was supported by "Læge Sofus Carl Emil Friis og hustru Olga Doris Friis' legat" and "Fonden til Lægevidenskabens Fremme".

Reference List

1. Boerma T, Ronsmans C, Melesse DY, Barros AJD, Barros FC, Juan L, et al. Global epidemiology of use of and disparities in caesarean sections. Lancet. 2018;392(10155):1341-8.

2. Betran AP, Torloni MR, Zhang J, Ye J, Mikolajczyk R, Deneux-Tharaux C, et al. What is the optimal rate of caesarean section at population level? A systematic review of ecologic studies. Reprod Health. 2015;12:57.

3. Molina G, Weiser TG, Lipsitz SR, Esquivel MM, Uribe-Leitz T, Azad T, et al. Relationship Between Caesarean Delivery Rate and Maternal and Neonatal Mortality. JAMA. 2015;314(21):2263-70.

4. Organization WH. WHO Statement on Caesarean Section Rates. Geneva, Switzerland; 2015.

5. Ramires de Jesus G, Ramires de Jesus N, Peixoto-Filho FM, Lobato G. Caesarean rates in Brazil: what is involved? BJOG : an international journal of obstetrics and gynaecology. 2015;122(5):606-9.

6. Cho CE, Norman M. Caesarean section and development of the immune system in the offspring. Am J Obstet Gynecol. 2013;208(4):249-54.

7. Morrison JJ, Rennie JM, Milton PJ. Neonatal respiratory morbidity and mode of delivery at term: influence of timing of elective caesarean section. Br J Obstet Gynaecol. 1995;102(2):101-6.

8. Yang XJ, Sun SS. Comparison of maternal and foetal complications in elective and emergency caesarean section: a systematic review and meta-analysis. Arch Gynecol Obstet. 2017;296(3):503-12.

9. Curran EA, O'Neill SM, Cryan JF, Kenny LC, Dinan TG, Khashan AS, et al. Research review: Birth by caesarean section and development of autism spectrum disorder and attention-deficit/hyperactivity disorder: a systematic review and meta-analysis. J Child Psychol Psychiatry. 2015;56(5):500-8.

10. Curran EA, Khashan AS, Dalman C, Kenny LC, Cryan JF, Dinan TG, et al. Obstetric mode of delivery and attention-deficit/hyperactivity disorder: a sibling-matched study. Int J Epidemiol. 2016;45(2):532-42.

11. O'Neill SM, Curran EA, Dalman C, Kenny LC, Kearney PM, Clarke G, et al. Birth by Caesarean Section and the Risk of Adult Psychosis: A Population-Based Cohort Study. Schizophr Bull. 2016;42(3):633-41.

12. Dalla Costa G, Romeo M, Esposito F, Sangalli F, Colombo B, Radaelli M, et al. Caesarean section and infant formula feeding are associated with an earlier age of onset of multiple sclerosis. Mult Scler Relat Disord. 2019;33:75-7.

13. Kristensen K, Henriksen L. Caesarean section and disease associated with immune function. J Allergy Clin Immunol. 2016;137(2):587-90.

14. Black M, Bhattacharya S, Philip S, Norman JE, McLernon DJ. Planned Caesarean Delivery at Term and Adverse Outcomes in Childhood Health. Jama. 2015;314(21):2271-9.

15. Sevelsted A, Stokholm J, Bonnelykke K, Bisgaard H. Caesarean section and chronic immune disorders. Pediatrics. 2015;135(1):e92-8.

16. Thapa B, Thapa A, Aryal DR, Thapa K, Pun A, Khanal S, et al. Neonatal sepsis as a major cause of morbidity in a tertiary center in Kathmandu. JNMA J Nepal Med Assoc. 2013;52(192):549-56.

17. Marild K, Stephansson O, Montgomery S, Murray JA, Ludvigsson JF. Pregnancy outcome and risk of celiac disease in offspring: a nationwide case-control study. Gastroenterology. 2012;142(1):39-45.e3.

18. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in new-borns. Proc Natl Acad Sci U S A. 2010;107(26):11971-5.

19. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. Nature. 2019.

20. Sandall J, Tribe RM, Avery L, Mola G, Visser GH, Homer CS, et al. Short-term and long-term effects of caesarean section on the health of women and children. Lancet. 2018;392(10155):1349-57.

21. Alur P. Sex Differences in Nutrition, Growth, and Metabolism in Preterm Infants. Front Pediatr. 2019;7:22.

22. Hassold T, Quillen SD, Yamane JA. Sex ratio in spontaneous abortions. Ann Hum Genet. 1983;47(1):39-47.

23. Di Renzo GC, Rosati A, Sarti RD, Cruciani L, Cutuli AM. Does foetal sex affect pregnancy outcome? Gend Med. 2007;4(1):19-30.

24. Norgaard-Pedersen B, Hougaard DM. Storage policies and use of the Danish New-born Screening Biobank. J Inherit Metab Dis. 2007;30(4):530-6.

25. Bliddal M, Broe A, Pottegard A, Olsen J, Langhoff-Roos J. The Danish Medical Birth Register. Eur J Epidemiol. 2018;33(1):27-36.

26. Mainz J, Hess MH, Johnsen SP. The Danish unique personal identifier and the Danish Civil Registration System as a tool for research and quality improvement. Int J Qual Health Care. 2019;31(9):717-20.

27. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem. 2005;51(10):1854-66.

28. Holm S. A Simple Sequentially Rejective Multiple Test Procedure. Scandinavian Journal of Statistics. 1979(6):65-70.

29. Wickham H FR, Henry L, Müller K. dplyr: A Grammar of Data Manipulation 2019 [Available from: https://github.com/tidyverse/dplyr.

30. Wickham H CW, Henry L, Pedersen TL, Takahashi K, Wilke C, Woo K, Yutani H ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics https://CRAN.R-project.org/package=ggplot22019 [Available from: https://github.com/tidyverse/ggplot2.

31. Malamitsi-Puchner A, Protonotariou E, Boutsikou T, Makrakis E, Sarandakou A, Creatsas G. The influence of the mode of delivery on circulating cytokine concentrations in the perinatal period. Early Hum Dev. 2005;81(4):387-92.

32. Werlang ICR, Mueller NT, Pizoni A, Wisintainer H, Matte U, Costa S, et al. Associations of birth mode with cord blood cytokines, white blood cells, and new-born intestinal bifidobacteria. PLoS One. 2018;13(11):e0205962.

33. Bellieni CV, Liuzzo LP, Giomi S, Tei M, Stazzoni G, Bertrando S, et al. C-reactive protein: a marker of neonatal stress? J Matern Foetal Neonatal Med. 2014;27(6):612-5.

34. Perrone S, Lotti F, Longini M, Rossetti A, Bindi I, Bazzini F, et al. C reactive protein in healthy term new-borns during the first 48 hours of life. Arch Dis Child Foetal Neonatal Ed. 2018;103(2):F163-f6.

35. Martikainen MV, Keski-Nisula L, Jakupovic H, Karvonen AM, Pekkanen J, Hirvonen MR, et al. The lack of natural processes of delivery and neonatal intensive care treatment lead to impaired cytokine responses later in life. Am J Reprod Immunol. 2017;77(3).

36. Christensen N, Sondergaard J, Christesen HT, Fisker N, Husby S. Association Between Mode of Delivery and Risk of Infection in Early Childhood: A Cohort Study. Pediatr Infect Dis J. 2018;37(4):316-23.

37. Daugaard AE HK, Hein M, Helmig RB, Hornshoj VG, Houman I. Antibiotika. http://gynobsguideline.dk/sandbjerg/120425%20ANTIBIOTIKA%20endelig%2025%204%2012.pdf; 2012.

38. Tribe RM, Taylor PD, Kelly NM, Rees D, Sandall J, Kennedy HP. Parturition and the perinatal period: can mode of delivery impact on the future health of the neonate? J Physiol. 2018;596(23):5709-22.

39. Pockley AG. Heat shock proteins as regulators of the immune response. Lancet. 2003;362(9382):469-76.

40. Ikeda K, Onimaru H, Matsuura T, Kawakami K. Different impacts on brain function depending on the mode of delivery. Brain Res. 2019;1720:146289.

41. Skogstrand K, Hagen CM, Borbye-Lorenzen N, Christiansen M, Bybjerg-Grauholm J, Baekvad-Hansen M, et al. Reduced neonatal brain-derived neurotrophic factor is associated with autism spectrum disorders. Transl Psychiatry. 2019;9(1):252.

42. Llurba E, Crispi F, Verlohren S. Update on the pathophysiological implications and clinical role of angiogenic factors in pregnancy. Foetal Diagn Ther. 2015;37(2):81-92.

43. Pastore S, Mascia F. Novel acquisitions on the immunoprotective roles of the EGF receptor in the skin. Expert Rev Dermatol. 2008;3(5):525-7.

44. Xian CJ, Zhou XF. Roles of transforming growth factor-alpha and related molecules in the nervous system. Mol Neurobiol. 1999;20(2-3):157-83.

45. Pei Q-M, Jiang P, Yang M, Qian X-J, Liu J-B, Zheng H, et al. Upregulation of a disintegrin and metalloproteinase-33 by VEGF in human airway smooth muscle cells: Implications for asthma. Cell Cycle. 2016;15(20):2819-26.

46. Kim S-H, Pei Q-M, Jiang P, Liu J, Sun R-F, Qian X-J, et al. Upregulation of MUC5AC by VEGF in human primary bronchial epithelial cells: implications for asthma. Respir Res. 2019;20(1):282-.

47. Santus P, Radovanovic D, Chiumello DA. Mucins and Asthma: Are We Headed to the Revolutionary Road? J Clin Med. 2019;8(11):1955.

48. Mjelle AB, Guthe HJT, Reigstad H, Bjorke-Monsen AL, Markestad T. Serum concentrations of C-reactive protein in healthy term-born Norwegian infants 48-72 hours after birth. Acta Paediatr. 2019;108(5):849-54.

49. Wander K, Brindle E, O'Connor KA. C-reactive protein across the menstrual cycle. Am J Phys Anthropol. 2008;136(2):138-46.

50. Kupelian V, Chiu GR, Araujo AB, Williams RE, Clark RV, McKinlay JB. Association of sex hormones and C-reactive protein levels in men. Clin Endocrinol (Oxf). 2010;72(4):527-33.

51. Kight KE, McCarthy MM. Sex differences and estrogen regulation of BDNF gene expression, but not propeptide content, in the developing hippocampus. J Neurosci Res. 2017;95(1-2):345-54.

52. Ploug T, Holm S. Informed consent and registry-based research - the case of the Danish circumcision registry. BMC Med Ethics. 2017;18(1):53.

53. Amatuni GS, Sciortino S, Currier RJ, Naides SJ, Church JA, Puck JM. Reference intervals for lymphocyte subsets in preterm and term neonates without immune defects. J Allergy Clin Immunol. 2019.

54. Wessberg A. Management and women's experiences of pregnancies lasting more than 41 gestational weeks. http://hdl.handle.net/2077/60289; 2019.

55. Bhutta AT, Cleves MA, Casey PH, Cradock MM, Anand KJ. Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. Jama. 2002;288(6):728-37.

56. Wiingreen R, Greisen G, Svensson J, Hansen BM. Low gestational age at birth and difficulties in school-A matter of 'dose'. PLoS One. 2018;13(6):e0198482.

Table 1. Maternal and neonatal demographic data

Type of birth	Type of birth	Type of birth	Pre-labor cesarean section	Pre-labor
Gender	Gender	Gender	Male	Female
	Number (%) (n=7266)	Number (%) (n=7266)	379 (5.2%)	355 (4.6%)
Birth weight	Birth weight	Birth weight		
	Mean (95% CI)	Mean (95% CI)	3543 (3491-3594)	3326 (3271-
Maternal BMI	Maternal BMI			
	Mean (95% CI)	Mean (95% CI)	25.5 (24.9-26.1)	25.4 (24.7-2
Maternal age	Maternal age	Maternal age		
	Mean (95% CI)	Mean (95% CI)	32.6 (32.2-33.1)	31.9 (31.4-3
Gestational age (weeks)	Gestational age (weeks)	Gestational age (weeks)		
	37	37	52 (7.3%)	50 (7.0%)
	38	38	159 (22.3%)	150 (21.0%
	39	39	139 (19.5%)	120 (16.8%
	40	40	21 (2.9%)	7 (1.0%)
	41	41	6 (0.8%)	6 (0.8%)
	42	42	2 (0.3%)	2 (0.3%)
Age at sampling (days)	Age at sampling (days)	Age at sampling (days)		
	2	2	270 (37.8%)	234 (32.8%
	3	3	105 (14.7%)	98 (13.7%)
	4	4	4 (0.6%)	3 (0.4%)
PROM	PROM	PROM	2 (0.5%)	4 (1.1%)

Table 2. Analytical characteristics

	working range pg/ml	working range pg/ml	inter assay
Analyte	**low**	**high**	**CV%**
BDNF	61.5	40000	28.7
CRP	43.6	10000000	47.3
EGF	2.5	5000	12.2
HSP70	246.1	5000000	17.8
IL-18	0.2	5000	13.8
MCP-1	4.4	50000	22.4
NT-3	2.0	5000	16.9
S100B	28.8	50000	24.6
sTNF-R1	16.9	100000	11.9
VEGF	3.0	5000	9.5

Table/Figure Caption List

Table 2. Analytical characteristics

The lowest concentrations in the working range was calculated as the concentrations 2.5 standard deviations above the lowest points on the calibration curves, and the highest points was set as the highest calibration points.

Inter assay CV% was calculated from controls present on all the plates, in total 211 plates.

Figure 1. Biomarker levels depending on gestational age and delivery form

The biomarkers are shown as mean with 95% confidence interval, divided into gestational age and pre-labour,

in-labour caesarean section and vaginal delivery, respectively. *statistical significance between pre-labour caesarean section and vaginal delivery, #statistical significance between pre-labour and in-labour caesarean section for the specific week. The p-values are calculated as an overall difference.

Figure 2. Gender differences in biomarker levels depending on birth form

The biomarkers are divided into gender and grouped into pre-labour, in-labour caesarean section and vaginal delivery. The data is shown as mean concentrations with 95% confidence interval. *there is a significant difference between gender. P-values are calculated as an overall difference comparing males and females irrespective of delivery form.

Figure 3. Biomarker levels depending on gestational age on delivery

The figure shows the concentrations of the different biomarkers divided into gestational age, combined for all delivery forms. The data is plotted as the group mean with the 95% confidence interval as the whiskers. Data points with no letters in common are significantly different on a 5% level.