From the
DEPARTMENT OF CLINICAL SCIENCE,
INTERVENTION AND TECHNOLOGY
DIVISION OF PEDIATRICS

Karolinska Institutet, Stockholm, Sweden

MODE OF DELIVERY, EPIGENETIC MODULATION AND IMMUNE CELL FORMATION AT BIRTH

Titus Schlinzig

Karolinska Institutet

Stockholm 2018

All previously published papers were reproduced with permission from the publishers.

Cover: Gastone Novelli “Linea”, with permission from Archivio Gastone Novelli/Roma and the Peggy Guggenheim Collection/Venezia, Italy.

Published by Karolinska Institutet.

Printed by Eprint AB 2018

ISBN 978-91-7676-991-1

Karolinska Institutet

Institutionen för klinisk vetenskap, intervention och teknik, Enheten för pediatrik, Karolinska Institutet

Mode of delivery, epigenetic modulation and immune cell formation at birth

Akademisk avhandling

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i C1:87, Karolinska Universitetssjukhuset, Huddinge

Fredag 25 maj 2018, kl 10.00

av

Titus Schlinzig, MD

Huvudhandledare
Professor Mikael Norman
Karolinska Institutet
Institutionen för klinisk vetenskap, intervention och teknik
Enheten för pediatrik

Bihandledare
Professor Tomas Ekström
Karolinska Institutet
Institutionen för klinisk neurovetenskap

Docent Stefan Johansson
Karolinska Institutet
Institutionen för klinisk forskning och utbildning, Södersjukhuset

Fakultetsopponent
Professor Bo Jacobsson
Göteborgs universitet
Institutionen för kliniska vetenskaper
Avdelningen för obstetrik och gynekologi

Betygsnämnd
Professor Eric Herlenius
Karolinska Institutet
Institutionen för kvinnors och barns hälsa

Professor David Ley
Lunds Universitet
Institutionen för kliniska vetenskaper

Professor Catarina Almqvist Malmros
Karolinska Institutet
Institutionen för medicinsk epidemiologi och biostatistik

till Lena & August

Abstract

Background

Birth by Cesarean section (CS) has been associated with a greater risk for immune disorders like allergy, asthma, type 1 diabetes, celiac disease, inflammatory bowel diseases, and cancer later in life. Although elective CS continues to increase rapidly, it is unclear if and how it may contribute to future health and disease. Our aim was to investigate the influence of mode of delivery on the global epigenetic state in white blood cells (WBC) as well as global and genome-wide, locus-specific epigenetic state in hematopoietic stem/progenitor cells. Further, we tested whether surge of immune cell formation at birth is related to mode of delivery, taking other maternal and infant characteristics in account.

Objective and methods

Study I and II are observational cohort studies including 37 (16 elective CS) and 64 (27 elective CS) healthy infants born at term. Cord blood was sampled and in study I, blood sampling was repeated 3-5 days after birth. Global DNA-methylation was analyzed in leukocytes by luminometric methylation assay (LUMA). In study II and in addition to LUMA, genome-wide, locus-specific DNA-methylation analysis of hematopoietic CD34+ cells was performed after cell separation using the Illumina Infinium 450K platform.

In **study III,** we used a prospectively collected database, including information on maternal and infant characteristics of 6,014 healthy, singleton infants delivered in Stockholm, Sweden, with gestational age ≥ 35 weeks. The data was linked to blood levels of T-cell receptor excision circles (TREC) and κ-deleting recombination excision circles (KREC), determined 3-5 days after birth as part of a neonatal screening program for immune-deficiencies, and representing quantities of newly formed naïve T- and B-lymphocytes.

Results

Global DNA-methylation of leucocytes and hematopoietic stem cells (CD 34+) was higher in infants delivered by elective CS compared to those born vaginally. The genome-wide, locus-specific analysis identified 343 loci with a > 10 % (maximal 40 %) difference of DNA-methylation between the two groups. In vaginally delivered infants, the degree of DNA-methylation in three loci gradually diminished in relation to duration of labor.

Infants born by CS before labor had a 32 % increased risk of having TRECs within the lowest quintile. Low TREC was also independently associated with male gender, preterm birth at 35-36 weeks of gestation and infant being small for gestational age. Low KREC was associated with male gender, postterm birth at > 42 weeks and small for gestational age. Maternal characteristics exhibited no associations with levels of TREC or KREC in newborn infants.

Conclusions

Mode of delivery affected the epigenetic state of the neonatal WBC and hematopoietic stem/progenitor cells. In addition, delivery by elective CS was associated with lower T-lymphocyte formation in newborn infants. The significance and functional relevance of epigenetic differences and reduced birth-related surge in lymphocyte formation for future health in children and adults delivered by CS remains to be explored.

List of publications

I. Schlinzig T, Johansson S, Gunnar A, Ekström TJ, Norman M.

Epigenetic modulation at birth – altered DNA-methylation in white blood cells after Caesarean section.

Acta Pædiatrica, 2009: 1096–1099. doi:10.1111/j.1651-2227.2009.01371.x

II. Almgren M, Schlinzig T, Gomez-Cabrero D, Gunnar A, Sundin M, Johansson S, Norman M, Ekström TJ.

Cesarean delivery and hematopoietic stem cell epigenetics in the newborn infant: implications for future health?

American Journal of Obstetrics and Gynecology 2014, Volume 211, Issue 5, Pages 502.e1-502.e8, ISSN 0002-9378.

III. Schlinzig T, Johansson S, Stephansson O, Hammarström L, Zetterström RH, von Döbeln U, Cnattingius S, Norman M.

Surge of immune cell formation at birth differs by mode of delivery and infant characteristics—A population-based cohort study.

PLOS ONE, 2017: 12(9), e0184748. 21

Contents

List of Abbreviations

ACTB	Actin beta
AGA	Appropriate for gestational age
BMI	Body mass index
bp	Base pair
C	Constant segment
CD	Cluster of differentiation
CGI	CpG island
cj	Coding joint
CpG	Cytosine-phosphate-Guanine
CRP	C-reactive protein
CS	Cesarean section
DMP	Differentially methylated position
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
GA	Gestational age
GR	Glucocorticoid receptor
GREAT	Genomic Regions Enrichment of Annotations Tool
ICD	International classification of disease
ICR	Imprinting control region
Igf2	Insulin-like growth factor 2
KREC	κ-deleting recombination excision circles
LGA	Large for gestational age
LUMA	Luminometric methylation assay
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
ncRNA	Non-coding ribonucleic acid
PKU	Phenylketonuria
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SGA	Small for gestational age
SI	International system of units
Sj	Signal joint
TCR	T-cell receptor
TCRD	T-cell receptor delta
THF	Tetrahydrofolate
TREC	T-cell receptor excision circles
VD	Vaginal delivery
V(D)J	Variable(diversity)joining segments
WBC	White blood cell

1 Introduction

Cesarean section (CS) has become the most common surgical procedure in women of child-bearing age and the rates are increasing rapidly in many countries worldwide.

Short-term maternal and infant outcomes after CS are well described. The long-term consequences of this change in mode of childbirth are however still mostly unknown.

Compared to vaginal delivery (VD), CS delivered infants have an increased risk developing immune associated disorders later in life, such as asthma and allergies, type 1 diabetes, celiac disease and inflammatory bowel diseases, immune deficiencies, leukemia, and other malignancies affecting young people. Also, associations between CS and increased risk of obesity and autism have been reported. If and how CS could affect the health in the offspring is, however, still unclear as the underlying mechanisms are poorly understood.

Possible pathways and mechanisms explaining or contributing to the abovementioned associations that have been suggested in earlier studies include the altered bacterial colonization of the neonatal gut – "the hygiene hypothesis" – and the lack of birth stress induced by labor and preparing the fetus for the world outside the womb. In relation to reduced birth stress, we hypothesized that this may result in maladaptive immune activation through modified epigenetic regulation of gene expression in white blood cells.

In this thesis, I will present two studies on mode of delivery and epigenetic state of newborn, and one study on how various perinatal risk factors, including CS before labor, affect the establishment of differentiated immune cells at birth.

2 Background

2.1 Developmental origins of adult health and disease

The concept of developmental origins of adult disease has its background in the epidemiological findings by David Barker and colleagues. They discovered that low birth size is related to a greater risk of death from cardiovascular disease[1] and type 2 diabetes mellitus[2] in adult age. Several epidemiological and clinical studies have led to the conclusion that the risk of developing diseases in adulthood can be influenced not only by genetics and adult lifestyle factors, but also by environmental factors acting in early life, even before birth via developmental plasticity.[3, 4]

Intrauterine or early childhood events can cause an adaption to an environment that can be predicted by the means of the event. This is a key concept of the developmental origins of adult health and disease. The concept, called "predictive adaptive response",[3] is evolutionary important since the adaptive response will predict the future environment and therefore optimize phenotype, which in turn will promote survival. A faulty prediction in early life may lead to a discrepancy between expected and real environment, resulting in early shaping of a phenotype which will have more narrow physiological boundaries in future life and therefore an increased risk of disease. For instance, a nutritionally sparse fetal life will cause an adaption to a postnatal life lacking adequate resources. If nutrition is not sparse in the postnatal environment, an energy conserving phenotype programmed early in fetal life by predictive adaptive response could be unfavorable and more easily lead to obesity and the metabolic syndrome. The changes of the predictive adaptive response may be permanent if they occur during critical developmental periods. This is now supported by a large amount of clinical and experimental data.[4] There is also increasing evidence from experimental studies in animal and humans that early environmental factors act to alter physiology through epigenetic processes.[5]

In the agouti mouse model (viable yellow agouti (A(vy)) carrying a retrotransposon that regulates the agouti gene, normally expressed only in hair follicles), food poor in methyl supplements given to pregnant mice reduces the level of DNA-methylation in the retrotransposon which leads to an ectopic gene expression and changes the phenotype of the offspring into an obese diabetic mouse

with increased risk of cancer.[6] This experiment demonstrates that the fetal nutritional environment may cause a lasting change of the offspring´s phenotype by an epigenetic mechanism. The in utero environmentally induced phenotype and the levels of DNA-methylation were also found in the second generation of offsprings.[7]

The research group of Meaney and Szyf demonstrated that not only fetal nutrition but also adverse neonatal stress could result in an increased stress-sensitivity of the adult offspring.[8] Rat pups raised by mothers who provided affectionate care in form of licking and grooming the first days after birth showed a different and lower degree of DNA-methylation in the promoter region of the glucocorticoid receptor gene (GR) in the hippocampus and the GR expression was increased as compared to pups to less affectionate mothers. The pups reared under these neonatal conditions became less stress sensitive as adults than pups reared by less attentive mothers and these findings could be replicated in a study design with cross-fostering. Meaney and Szyf went on to study the homologous region of the hippocampal promoter in men who had committed suicide. Suicide victims with a history of abuse in childhood showed an increased DNA-methylation similar to that found in the rat experiment, whereas suicide victims without a history of child abuse did not.[9]

An increased level of DNA-methylation was also found in the same promoter region of the glucocorticoid receptor gene in mononuclear cord blood cells in newborn infants born by mothers with depressive symptoms in the third trimester.[10] Such DNA hypermethylation was associated with increased salivary cortisol levels after exposure to stress at 3 months of age.

A recently published review, including 23 studies, on the relationship between certain early-life exposures (early-life socio-economic circumstances, childhood obesity, and early-life nutrition) and DNA-methylation in human subjects, supports that epigenetics, specifically DNA-methylation, is a plausible mechanism through which early life environment can act and influence health outcomes years after the exposures.[11]

In summary, evidence is increasing for a number of different early-life events and exposures leading to epigenetic modifications that are long-lasting and therefore can have health-related consequences later in adult life.

2.2 The "stress" of being born

Being born is sometimes described as an affliction.[12] The human baby is driven through the birth canal often for many of hours, with the head exposed to substantial pressure. From a low starting point of arterial partial pressure of oxygen, the fetus is exposed to repeated hypoxic episodes along with uterine contractions

and may be at risk of asphyxia if the placental circulation or umbilical cord is depressed too much. From that perspective, birth stands in strong contrast to prenatal life. From a warm and protected environment, the newborn infant is delivered to a colder and much less protected environment outside.[12]

The birth can be described as "stressful" for the infant, but what do we know about the "stress" of being born?

The biological stress of being born surpass all other stress later in life *(Fig 1)*. It is fundamental for intact survival during the transition from fetal to neonatal life. The fetus and the newborn infant have large stores of catecholamines in the adrenal glands and in the paraganglia along the aorta.[13] Lagercrantz and Slotkin measured the levels of catecholamine in umbilical cord blood after normal VD. They discovered that the levels of catecholamines immediately after birth were much higher than in adults during different kinds of stressful activities. The levels of catecholamines after VD were even higher than in patients with pheochromocytoma, a catecholamine producing tumor *(Fig 1)*.

On the contrary, infants, delivered by elective CS, had only slightly elevated levels of catecholamines compared to resting adults.[14] After emergency CS, often after established labor, the catecholamine levels increased substantially, similar to those after VD. The findings suggest that the uterine contractions, together with some hypoxia, cause the release of catecholamines.[12] It is known that vaginally delivered infants adapt easier to extrauterine life, for example by start breathing more easily. This can be explained by the stimulation of adrenaline on the absorption of lung fluid.[13, 15] Infants delivered by elective CS show more often transient respiratory distress and their glucose levels are slightly lower,[16] see section 2.3.2. Another marker of stress, cortisol, measured in cord blood is significantly lower in infants delivered by elective CS.[17] Also, elective CS infants have a lower body temperature, probably due to decreased noradrenaline levels, resulting in less stimulation of brown fat that generates body heat.[12, 18] Labor activates the inflammatory defense systems[19] and the central nervous system[20] so that the

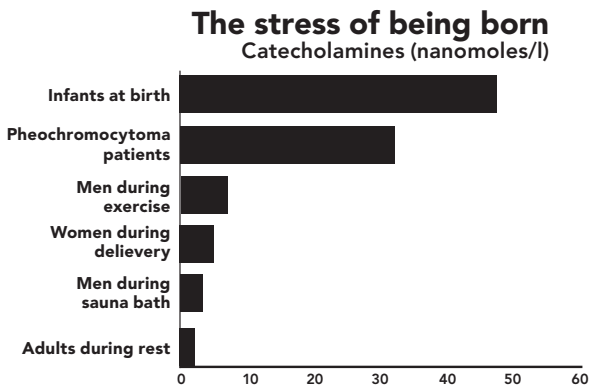

Fig 1.
Catecholamine levels in different situations in life, modified according to Lagercrantz and Slotkin.[13]

fetus is most favorable prepared and suited for life outside the uterus. Compared to normal VD, stress in infants delivered by elective CS is immediate rather than gradually developed as during labor. CS may therefore be maladaptive for the newborn infant.

In summary, the physiological release of catecholamines brings a lot of benefits for the newborn infant and we may therefore call the "stress" of being born a "good stress".[12]

2.3 Mode of delivery

The two principal modes of delivery are vaginal delivery and cesarean section. Of all the deliveries in 2015 in Sweden, there were 82.6 % VDs, of which 75.4 % were non-instrumental and 7.2 % instrumental, mainly vacuum extractions.[21] The most common indications for instrumental VD were prolonged labor, dystocia, or signs of fetal hypoxia. In 83.7 % of VD, the labor started spontaneously, whereas 16.3 % had an induced labor. Causes of induction were post-term gestation (duration of pregnancy exceeded 42 + 0 weeks), multiple pregnancy, unclear bleeding during pregnancy, declining fetal growth or emerging disease in the mother, e.g. preeclampsia or diabetes.[21] Uterine contractions were mainly started artificially by drug treatment, but amniotomy and the use of balloon catheters were also used.

In total, 17.4 % of pregnant women underwent CS, of which 7.8 % were elective and 9.4 % emergency CS.[21] Causes of planned/elective CS included breech position, preeclampsia, multiple birth after complicated pregnancy, placenta position impeding VD (placenta previa), psychosocial indication, former CS, small maternal pelvis, or large baby. Indications for emergency CS included complications during VD, such as elongated delivery, weakness of labor, severe bleeding, uterine rupture, and/or threatening asphyxia of the fetus.

2.3.1 Frequency of Cesarean section – international perspective

The rate of CS has quadrupled worldwide in less than two decades. Data from the first decade of the 21st century are presented in fig 2. That makes CS the most common surgical procedure performed in women of child-bearing age.[22-24]

Until the beginning of the 20th century CS was very uncommon.[25] In the period between 1926 and 1930 the CS rate in Sweden was 0.25 %.[26] Community hospitals in Detroit, USA, with more than 30 000 deliveries per year 1926 - 1930 performed CS in 0.45 % to 0.6 % of their deliveries.[25] During the period 1951 to 1980, CS rates increased from 1.7 to 11 % in Sweden.[26] In the last five years, Swedish CS rates have been stable around 17 – 18 %.[21]

CS has been estimated to be medically indicated in up to 20 % of all deliveries.[27, 28] However, many national CS-rates exceed this level, suggesting that many women probably undergo CS without a clear medical indication.[24, 29] *(Fig 2)* In 2009, Brazil was the first country where the rate of CS, comprising 50.1 % of all deliveries, surpassed the quantity of VD. The rate has since then continue to increase, with statistics from 2014 reporting a CS rate of 55.7 %.[30]

There are several potential causes for the increasing rates of CS:

- Changes in the risk profiles of the mother-to-be as maternal age, obesity and diabetes are considered to be important contributors for the rising rates of CS.[31, 32]
- Higher numbers of elective CS in first-time mothers may contribute to CS also in following deliveries.[30, 32]
- More multiple pregnancies resulting from in-fertility treatments and assisted reproduction may play a role.[33]
- Intense and very intense ("tocophobia") fear of spontaneous childbirth is the most common maternal request for elective CS.[32, 34]
- In many countries, a defensive, risk-orientated obstetric practice and the doctors´ insurance premiums may have an influence on the choice of mode of delivery, where elective CS is an attractive alternative.[32]
- A general increased tendency to risk avoidance and contemporary media flow are regarded playing an important part.[32]

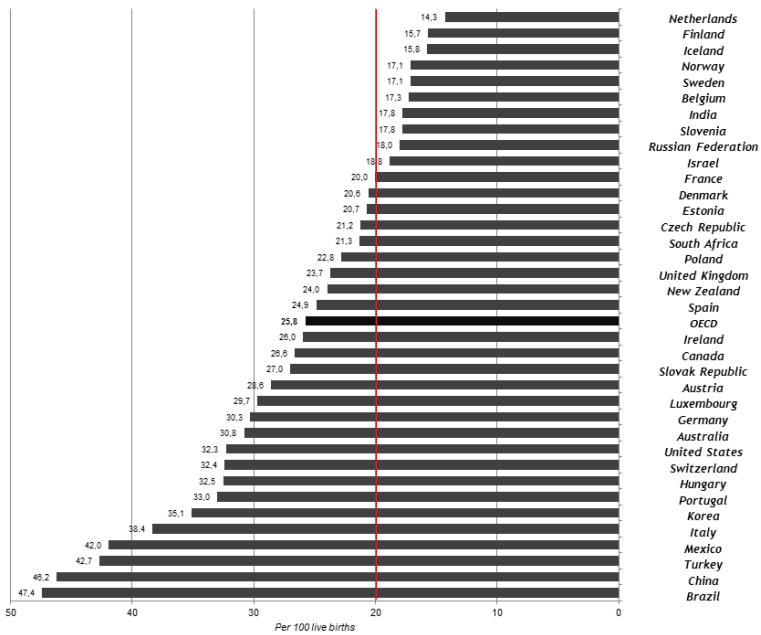

Fig 2. CS rates 2009 or nearest year before, modified according to OECD Health Data 2011; WHO (2008a).

2.3.2 Cesarean section, short-term consequences

The short-term consequences for the newborn infant delivered by elective and emergency CS are well characterized.[35-37] Compared to VD, there is an increased neonatal morbidity and mortality after CS. In the public opinion, it is not rarely assumed that CS prevents neonatal risks, because CS can be life-saving in certain situations. However, this is only true for seldom-occurring complications.[38]

The most common neonatal morbidity after elective CS is adverse respiratory outcome. It often appears as transient tachypnea of the newborn, but it also occurs as respiratory distress syndrome with need of mechanical ventilation.[36, 37] Other adverse neonatal outcomes associated with an increased risk after CS are:

- admission to the neonatal intensive care unit
- need for cardiopulmonary resuscitation
- mechanical ventilation within 24 hours after delivery
- delayed adaption from fetal to neonatal circulation with persisting pulmonary hypertension
- newborn sepsis
- hypoglycemia
- prolonged hospitalization[36, 39]

The lowest morbidity and mortality is found in the gestational weeks 38 to 40.[36, 37] Nevertheless, the adjusted risk for mortality was doubled for infants delivered by elective CS compared to those born by normal VD.[37] If CS is needed due to reasons like breech position, multiple childbirth, placenta previa, psychosocial indication, former CS, small maternal pelvis, or large baby, near-term would be the best timing to be recommended.

2.3.3 Cesarean section, long-term outcomes

Several studies have shown that CS delivery is associated with an elevated risk of adult-onset immune disorders, neuropsychiatric conditions and obesity compared to VD.[40-42] Many of the studies on the long-term differences between elective CS and VD are prospective, population-based and adequately adjusted for known confounders. The epidemiological findings that indicate a long-term impact of CS on the risk of adult-onset diseases may be limited. This is due to the heterogeneity of study results arisen from confounding variables hard to control for. These include use of anesthetic agents, antibiotics given, diet, hospital environment, and the genetic diversity of the participants.[40]

Also, another important limitation in many studies is the difficulty to discriminate between elective prelabor CS and emergency CS after onset of labor. With this limitation follows a possible underestimation of the effect of elective

prelabor CS, since some studies confined an association between the risk of disease later in life and elective CS.[40, 43, 44]

Cesarean section and risk for morbidity in later life

In a meta-analysis of 23 studies, Thavagnanam et al could demonstrate that individuals delivered by CS have a 20 % higher risk of developing asthma, both as children and adults, compared to those born vaginally.[45] After adjustment for known confounders, such as maternal smoking, low birthweight, and duration of breast-feeding, the association was still consistent. A second meta-analysis by Huang et al, based on 26 studies, found a risk of 21 % after elective and 23 % after emergency CS for asthma later in life.[46] Several possible explanations for the increased risk for asthma have been discussed, such as preterm birth, lower birthweight, the hygiene hypothesis, prophylactic antibiotics, less breast-feeding and epigenetic modifications. A large Danish register study analyzed children born at term by CS under a 35-year period and found a 23 % increased risk for asthma.[47]

Allergic rhinitis and atopy have been reported to be more prevalent in children born by CS.[48, 49] There is evidence that children delivered by CS have an increased risk of developing sensitization to food, although not all the studies could confirm the association.[48, 50, 51]

Furthermore, a meta-analysis including 20 studies has shown that children born by CS have a 23 % higher risk of developing childhood-onset type 1 diabetes compared to infants that are born vaginally.[52] This association was not modified when it was adjusted for confounders such as maternal age, birthweight, gestational age, birth order, breast-feeding, maternal diabetes, or family history of diabetes.

Increased hospitalization rate due to gastroenteritis in early childhood has been related to birth by CS.[53] A greater risk for celiac disease has been found in children born by elective CS[43] and CS totally[54] compared to VD. In the Danish register study there was an 20 % higher risk for inflammatory bowel disease.[47]

Indications for an elevated risk of cancer, such as leukemia, neuroblastoma, and testicular cancer in children and young adults born by CS compared to VD [47, 55-58] were described, certain leukemia even particularly in prelabor CS.[57] Other studies could not report associations.[59, 60]

Furthermore, a higher risk of 46 % for immunodeficiency diseases has been detected in children and adults delivered by CS.[47]

Also, systemic connective tissue disorders and juvenile arthritis were associated with delivery by CS.[47]

Delivery by CS has been associated with a 36 % increased risk for aseptic necrosis of the femoral head (Legg-Calvé-Perthes disease) later in childhood, also after adjustment for other risk factors such as breech position.[61]

Two meta-analyses, containing 9 and 28 studies, indicate a 33 and 34 % higher risk of obesity in childhood and adolescence, if the participants were born by CS.[42, 62] Some studies regarding the risk of developing childhood obesity could find a difference between elective CS and emergency CS,[63] other not.[64, 65]

Additionally, previous studies have examined possible connections between CS and autism spectrum disorders with conflicting findings.[41] A Finnish case-control study suggests a possible association between autism and elective prelabor CS but not emergency CS.[44]

2.3.4 Bacterial gut colonization – the hygiene hypothesis

The "hygiene hypothesis" is one theory about underlying causes for the higher risk of developing immune-related diseases later in life after delivery by CS. It suggests that a too clean environment, especially in early childhood, may contribute to a greater risk of developing diseases related to the immune system, such as atopic diseases. It was proposed for the first time by Strachan, who observed an inverse correlation between hay fever and the number of older siblings.[66]

Newborn babies delivered vaginally are colonized by bacteria from the mother's birth canal and perianal region and the exposure to the bacteria may form their immune development.[67] Newborns delivered by prelabor elective CS have an improper bacterial exposure and are predominantly colonized by bacteria originating from the hospital environment.[68] They are also separated from their mothers for a longer time, resulting in delayed breast-feeding, altering the bacterial colonization and growth in the neonatal gut.[69] The divergent intestinal colonization of infants born by CS may lead to abnormal neonatal immune responses, elongate postnatal immunological maturity processes, inhibit competent immunological priming, and enlarge the risk for later immune disease.[70]

2.4 Epigenetics

Conrad Waddington (1905-1975) is credited with coining the term "Epigenetics" in 1942 as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being".[71]

Epigenetics supply mechanisms for the functioning genome and mediate adaptations to a dynamic and changeable environment.[72-74] Every human cell contains about 20000 to 30000 regulated genes.[75] Only a subset is expressed in any given cell. The expression is controlled by genome-wide epigenetic mechanisms.

Today, the most accepted definition of epigenetics is "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence".[76, 77] Strictly considered, it would only be DNA-methylation. But commonly, in addition to DNA-methylation, histone

modification and non-coding RNA are also regarded as epigenetic mechanisms.[72-74, 77, 78]

2.4.1 DNA-methylation

DNA-methylation is the most basic and best studied form of epigenetic modification. DNA-methylation is a process by which methyl groups are added to cytosine in the DNA molecule forming methylcytosine, overwhelmingly most common in the context of cytosine-guanine dinucleotide (CpG). Cytosine (C) is followed by a guanine (G), held together by a phosphodiester bond (p).

In mammalian cells methylation occurs almost only on the fifth position of cytosine *(Fig 3)*. Homocysteine, folate and cofactor cobalamin (B12) play an important role for the methylation of cytosine *(Fig 3)*. In a reaction with methionine synthase, 5-methyltetrahydrofolate is transformed into tetrahydrofolate (THF), while transferring a methyl group to homocysteine and transforming it to methionine. Methionine reacts with adenosine triphosphate to form S-adenosylmethionine, driven by the methionine adenosyltransferase. S-adenosylmethionine act as a donor of the methyl group (CH3) to cytosine (and other acceptors such as histones and other proteins).

Methylation of cytosine does not change the DNA sequence.[79] The two conceptionally different variants of DNA-methylation are "maintenance" and "de novo" methylation. Patterns of methylation are maintained through cell division and DNA replication by the maintenance methyltransferase DNA (cytosine-5)-methyltransferase 1 (DNMT1), one of the so far known three mammalian DNA methyltransferases.[79] Methylation "de novo" is independent of DNA replication and occurs i.e. in early embryogenesis and development. "De novo" methylation is catalized by the other two DNA methyltransferases DNMT3A and DNMT3B.[79, 80]

The human genome contains approx. 28 million sites (CpG-sequences),[81]

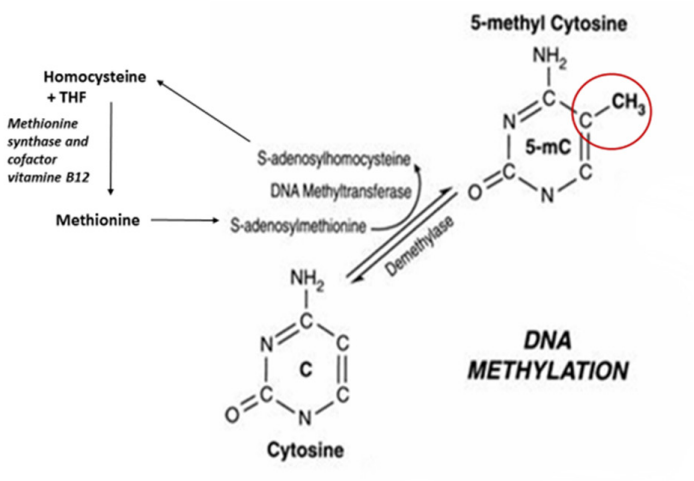

Fig 3. DNA-methylation of cytosine at the fifth position with S-adenosylmethionine as methyl donor, THF: tetrahydrofolate.

where cytosine can potentially be methylated to e.g. produce specific cell lineages during development and in response to environmental exposures. Methylation patterns are thus specific for every cell type and allows or prevents gene transcription. DNA-methylation of cytosine is generally associated with transcriptional silencing when it occurs in gene promoters *(Fig 4).*[79, 82] However, enhancer regions are also regulated by DNA-methylation and methylation of cytosine may allow or prevent gene transcription depending on context. In the mammalian genome, 60–80% of CpG sites are generally methylated.[81] The DNA-methylation in mammalian DNA is not evenly distributed. Regions with a high frequency of CpG sites are called CpG islands. CpG islands usually encompass a region with at least 200 base pairs (bp) of densely populated CpG sites.[83] About 10 % of all CpG sites are located in islands.[84] Computationally, it is estimated that the human genome contains 29,000 CpG islands and about 60 % of human genes are associated with CpG islands.[85] Around 70 % of CpG islands are located in gene promoter regions.[86] CpG islands are predominant at transcription start sites of developmentally regulated genes and they are under normal conditions mostly unmethylated.[84]

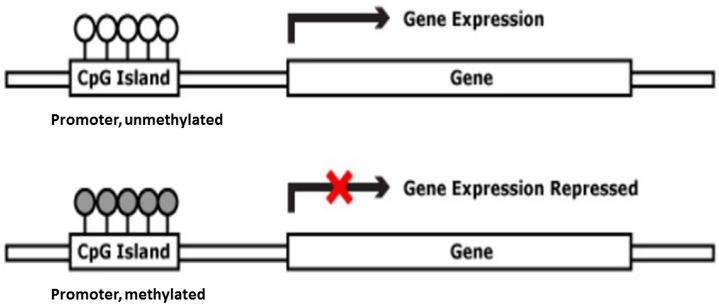

Fig 4. DNA-methylation in promoter CPG island preserves activation, modified according to Métivier et al .[82]

One example of DNA-methylation in a promotor region is the exon 1_7GR promoter region of the hippocampal glucocorticoid receptor gene in rat offspring, already mentioned in section 2.1.[8] Rat pups raised by mothers who gave them better care in form of more licking and grooming were less stress sensitive. The expression of the GR mRNA is increased in the offspring of "good care taking" mothers or after manipulations that increase maternal licking and grooming, due to increased transcription of the hippocampal GR gene, coding for GR. The activity of this promoter is enhanced due to maternal care.[8, 87] Analysis of the individual methylated cytosines within CpGs of the exon 1_7GR promoter in hippocampus showed significant methylation differences in some of the 17 cytosines of the exon 1_7GR promoter depending on pup licking and grooming behavior by the

rat mothers.[8] DNA-methylation was significantly lower in the offspring of "good care taking" mothers. This agrees with findings that decreased methylation of CpGs in regulatory regions of genes is associated with active chromatin structure and transcriptional activity.[88]

Enhancers are short (50-1500 bp) DNA elements that an activator (transcription factor) can bind to and regulate the transcription of a certain gene.[89] They are often located within the intron of the genes or neighboring genes and seldom near to the promoter.[90] The supposed mechanism for enhancer function on promoters is a direct interaction through "looping" the DNA, allowing an enhancer-promoter interaction.[90] Proteins, e.g. transcription factors, are bound to both enhancer and promoter to start transcription when enhancer and promoter are located close to each other. Meanwhile methylation of CPG islands in promoter regions mostly correlates to a silenced gene,[84] can methylation of enhancers result in activation of gene transcription.[90]

One example is the methylated imprinting control region (ICR) in the enhancer of the *Igf2* gene (Insulin-like growth factor 2), a growth promoting hormone during gestation.[91] Every cell contains maternal and paternal DNA/alleles. In the paternal allele is the ICR of the enhancer methylated and the transcription of the gene active. The maternal enhancer ICR is bound by CTCF (CCCTC-Binding Factor or Zinc finger protein), which acts as an insulator (regulatory element), preventing downstream enhancers from looping to and activate the *Igf2* promoter on that allele.[91] Methylation of the ICR on the paternal allele prevents CTCF from binding and acting as an insulator, and thus allows the downstream enhancers to activate the *Igf2* promoter. The methylation of the ICR on the paternal allele also spreads into the *H19* gene promoter and silencing it *(Fig 5)*.

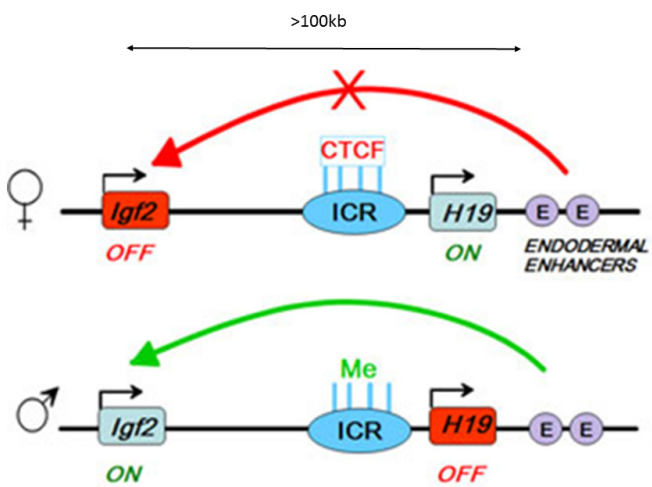

Fig 5. Activation of the paternal Igf2 by methylation, modified according to Bell et al .[91]

Methylated cytosine can be enzymatically oxidized to hydroxymethylcytosine by the catalyzer Tet protein *(Fig 6)*. DNA methyltransferases cannot recognize hydroxymethylcytosine, and DNA-methylation may be lost at CpG sites during DNA replication.[92] It can also be further oxidized and eventually replaced to cytosine in the absence of replication. The significance of 5-hydroxymethylcytosine is still poorly understood. However, of all tissues the highest level of hydroxymethylcytosine is found in the human brain, up to 1 % of all cytosines in the human brain cortex are hydroxymethylated.[93] It has been suggested that it could be an epigenetic modification by itself that may direct transcription through forming chromatin structure and allowing transcription.[92] The percentage of hydroxymethylcytosine is much lower in WBCs, and the genome-wide measurement of DNA-methylation by Illumina 450K (one of the methods, we applied) cannot distinguish between methylcytosine and hydroxymethylcytosine.

The methods of gene-specific and global DNA-methylation measurement, we used, are described in chapter 4.2.

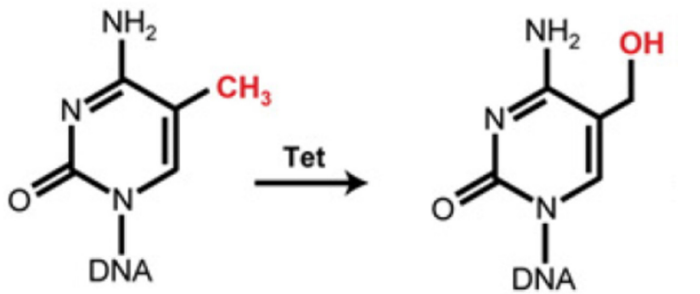

Fig 6.
Oxidation of methylcytosine to hydroxymethylcytosine catalized by Tet protein, modified according to Hahn et al .[92]

2.4.2 Histone modification

The other major epigenetic mechanism involves histone modifications, which in close interaction with DNA-methylation regulate chromatin structure and transcription of the genome. Histones are highly alkaline proteins in eukaryotic cell nuclei. They form structural units called nucleosomes, which package and order the DNA into building blocks of chromatin.[74] Chromatin is the complex of nucleosomes that in their most compact form is called heterochromatin and in a "beads on a string" structure euchromatin *(Fig 7)*. Eight subunits of histones, two each of H2A, H2B, H3, and H4 histones form the nucleosome. DNA wraps around the nucleosomes with approximately 147 base pairs *(Fig 7)*. The compaction of DNA contributes to structural support as well as serves functional roles.[94] Well-studied mechanisms of histone modification that occur at the histone tails

are acetylation, phosphorylation, and ubiquitination, which influences coiling of DNA around histones *(Fig 7)*.

Acetylation and deacetylation take place on the amino group of lysine by histone acetyltransferases (HATs) and histone acetyldeacetylases (HDACs), respectively.[95] Through phosphorylation, a phosphate group is added to the amino acids serine, threonine, or tyrosine of the histone tail.[96]

Ubiquitin is a small protein, consisting of 76 amino acids, first described 1975 by Goldstein.[97] Histone H2A was the first protein identified to be modified by ubiquitin. Ubiquitin is added to lysine, often as a single molecule, but also as multiple entities in ubiquitin chains. They are essential for normal cellular functions but the detailed mechanisms are still not completely understood.[98]

All these modifications control the packing of DNA in the nucleosomes and consequently influence the activity of transcription of genes.[74, 85]

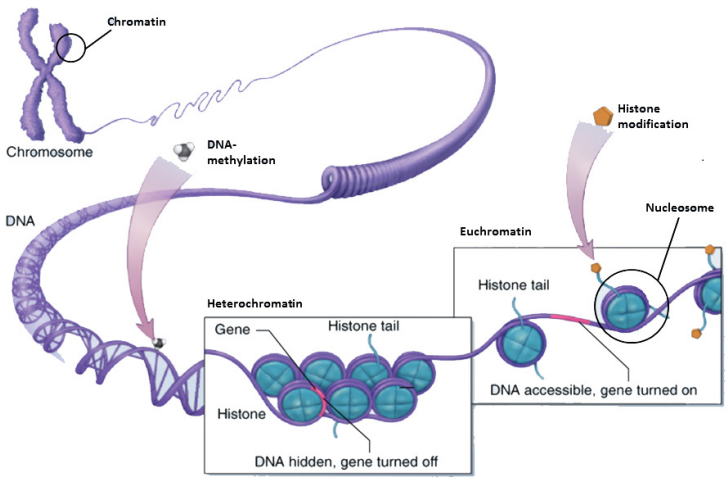

Fig 7. Chromosome, genes, DNA, histone modification and methylation, modified according to Oropello et al .[99]

2.4.3 Non-coding RNA

About 90 % of the human genome is transcribed, but only 1–2 % of these transcripts encode proteins, whereas the majority are transcribed as non-coding RNA (ncRNA).[78]

Epigenetic function of different subclasses of ncRNAs are chromatin formation (packing of DNA) and regulation of gene expression at transcriptional or posttranscriptional level.[78] For example, the subgroup of small interfering RNA affects the formation of heterochromatin directly. Another subgroup, microRNAs, mediates posttranscriptional gene silencing by binding to mRNA and preventing translation,[100] and probably regulates gene expression also at the direct level of transcription.[101]

There is substantial ongoing research about ncRNA. Some of their mechanisms are well described and studied, others are still poorly understood.[78]

2.5 Immune system and the newborn infant

The immune system is the body´s defense system that protects against microbiologic threat, malignant cells, and other pathogens. Immunity is divided into two parts, the innate and the adaptive responses. The major difference is how fast and specific they may act.

The term innate immunity implies immediate host defense. Parts of the innate immune system are neutrophils, monocytes, macrophages, complement system, cytokines, and acute phase proteins. Innate immunity is unspecific and can even be found in the simplest animals.[102]

The adaptive immunity is distinguishing for the immune system of higher developed animals. Parts of the adaptive immunity are T- and B-lymphocytes, reacting antigen-specific. The adaptive response takes time, several days or weeks to develop, but is specific. The adaptive response develops memory, so that the following exposure leads to a more rapid response, although not immediate.[102]

Innate response

Neutrophil recruitment to the site of infection is a first major step in the innate reaction.[103] They are released from the stimulated bone marrow into the circulation and other body compartments and cause the so-called "classic" leukocytosis. The complement system, containing about twenty regulatory glycoproteins, forms transmembrane proteins in pathogen's cell membrane and that leads to death by osmotic lysis and enhances other parts of the immune response, i.e., inflammation and opsonisation.[102] Other parts of the innate response are eosinophils protecting the host from parasitic infections, mast cells and basophiles, involved in severe immunological reactions, such as anaphylaxis and angioedema.[102] Additionally, natural killer cells recognize abnormal cells and are alike lymphocytes but lacks specific antigen receptors. They are programmed to lyse the target, e.g. virus-infected cells.[102] Finally, adhesion molecules, cytokines, and interferons are part of the cellular communication to work effectively where they are needed.

Adaptive response

Specific immunity consists mainly of T- and B-lymphocytes and their subpopulations, functioning antigen-specific. The first step is the antigen presentation to and recognition by the antigen-specific T- or B-lymphocyte. That leads to cell priming, activation, and differentiation, and usually occurs in lymphoid tissues. The second step is effector response, either the activated T-lymphocyte leaves the lymphoid tissue and targets the disease site or the activated B-lymphocyte, also called plasma cell, releases antibodies into the blood and tissue fluids.[102] The development and formation of antigen-specificity and receptor rearrangement will be described in the next chapter, because it is prerequisite for TREC and KREC

measurement.

Antigen presentation occurs in two ways. Antigens produced inside the cell (endogenously) form a complex with HLA/MHC class I molecules. Exogenous antigens (located outside the cell) are processed by special antigen-presenting cells, such as dendritic cells, B-cells, and macrophages. They are processing the antigen via different pathways inside the cell and express the antigen with MHC class II molecules. The two major types of effector T-lymphocytes are T helper (Th CD 4+) and T cytotoxic (Tc CD 8+) cells. CD 4+ lymphocytes detect only antigen presented by MHC class II and CD 8+ lymphocytes antigen presented by MHC class I. Th- and Tc-cells are functionally divided in subtypes Th 1/Th 2 and Tc 1/ Tc 2, and they can be distinguished by the cytokines they produce.[102]

B-lymphocytes produce different classes of antibodies, immunoglobulins (Ig). IgM is mainly found intravascular; IgG is the main antibody of the blood and tissues and IgA is especially found in mucosa secretions. The immunoglobulins serve to neutralize toxins, prevent organisms from adhere to mucosal surfaces, activate complement, opsonize bacteria for phagocytosis, and sensitizes infected cells for antibody dependent cytotoxic attack by killer cells.[102]

Immune system in the neonate

Neonates have confined immunological memory and the difference to adults is a still developing immune system. They are more exposed to infectious factors and agents as their adaptive immune response is suboptimal.

Newborn infants have received placentally transferred maternal antibodies, IgG´s, which contribute to early defense against pathogenic microorganisms. This passive protection by maternal antibodies is after all short lived and diminishes over the first 6 months of life.[104]

However, diseases affecting the mother during pregnancy, like allergy, infections, the mother´s nutritional state, and breastfeeding can have an impact on the developing immune system of the neonate and infant.[104] For example, nutritional stress in mothers leads to increased level of the hypothalamic-pituitary-adrenal hormone and hence the following fetal exposure to lower thymic weight and reduced levels of cortical lymphocytes. It also activates an endogenous endonuclease, which causes thymocyte apoptosis and altered B- and T-cell development.[105]

The innate immune response for protection against infections is very important in neonates, mostly due to their limited adaptive immune system.[106] Neutrophils are a major part of the innate response, but they have poorer function, grade of amplification and mobilization[104] and make neonates particularly liable to sepsis. Though there is a higher count of neonatal natural killer cells they are inferior in their function.[107] The same applies for antigen-presenting cells.

According to Basha et al,[104] the available data about adaptive immune response indicate a differential programming and functioning of neonatal T- and B-lymphocytes, compared to those of adults. Overall, it is characterized by an immaturity of their lymphocytes, a high presence of naïve recent thymic emigrants, low numbers of effector-memory T-cells, impaired Th 1 cytokine secretion and less strength of B-cell receptor signaling.

In summary, newborn infants have a higher vulnerability for infections due to a developing adaptive response compared to older children and adults. On the other side, they have a somewhat more efficient innate response and a passive immune protection through maternal antibodies, especially against virus. Additionally, breastmilk contains immunomodulatory cells, cytokines, exosomes and lactoferrin that help to protect newborns and infants from viral and fungal infections.[104, 108]

2.6 Hematopoietic stem cells, naïve T-and B-lymphocytes, TREC and KREC

The development of hematopoietic stem cells during embryogenesis is a complex process and occurs in multiple anatomical sites, such as the yolk sac, the aorta-gonad-mesonephros region, the placenta, and the fetal liver.[109] From the middle of the second trimester, they colonize the bone marrow. In adults, all classes of blood cells are derived from bone marrow resident hematopoietic stem cells. The hematopoietic stem cells give rise to the blood cells through the process of hematopoiesis.[110]*(Fig 8)* Existing data from previous studies support the role of epigenetic mechanisms including DNA-methylation in the control of gene expression in hematopoiesis.[111-114]

T- and B-lymphocytes develop from the common lymphoid progenitor in the bone marrow. B-lymphocytes remain in the marrow for their development, while T-lymphocytes migrate to the thymus. Both cell types develop antigen specific receptors. That happens through a process of random rearrangement and splicing of multiple DNA segments, coding for antigen binding areas.[102] The rearrangement arise early in the development of lymphocytes and leads to 10^8 T-cell receptors and 10^{10} antibody specificities.[115]

A description of the T-cell receptor rearrangement and formation of T-cell receptor excision circles (TREC) follows in this passage, but the mechanism is similar for the B-cell receptor.

T-lymphocyte differentiation occurs in the thymus and is characterized by rearrangement steps in the T-cell receptor (TCR) genes, resulting in the joining of variable (V), diversity (D), joining (J) and constant (C) gene segments,[116] in the literature called for V(D)J rearrangement or recombination *(Fig 9)*.

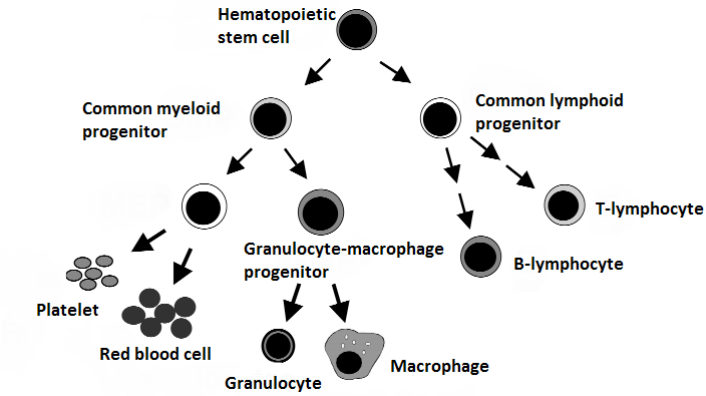

Fig 8. Schematic of hematopoietic development, modified according to Forsberg et al.[110]

The T-lymphocyte receptor exist in two forms, the most common (> 85 %) is a heterodimer of an α and β chain with a constant and variable domain, a minority of T-cells express an alternate receptor, formed by variable γ and δ chains.[102] During each of the rearrangement steps, DNA fragments are deleted as circular excision products, TRECs. They are excision products of the gene rearrangement in maturing thymocytes.[116] TRECs are found in thymocytes and mature T-lymphocytes, but their role in the cell is not totally clarified. TRECs are assumed to have a high over-time stability, they cannot multiply and consequently are diluted during T-cell proliferation.[116] The δRec-φJα TREC, is a late TCRD (T-cell receptor delta chain) deletion, produced by 70 % of developing human T-cells that express αβ TCRs.[116, 117] The quantitative PCR amplification across the joined ends of the δRec-φJα TREC, that we used in paper III, are regarded to reflect the number of recently formed naïve T-cells. TREC containing naïve T-lymphocytes are already produced in the fetal thymus, but the highest output occurs at birth and at the end of the first week of postnatal life, around three-quarters of the T-cells are expected to be produced during or after delivery.[118] It should also be noted that TREC containing T-lymphocytes can be very long-lived,[119] and are not entirely representing recently formed T-cells by the thymus in later life.

During B-cell maturation, immunoglobulin κ-deleting recombination excision circles (KRECs) are produced, similar to the excision of TREC in the rearrangement in T-lymphocytes.[102] Two kind of KRECs are produced, the coding joint (cj) KREC, which remains within the chromosome, whereas the signal joint (sj) KREC is excised from genomic DNA. The (cj)KREC levels remain the same after B-cell division, whereas (sj) KRECs are not replicated during cell division.[121] (sj) KRECs are a measure of newly matured naïve B-lymphocytes.

Determination of TREC and KREC levels is for example performed together with the neonatal screening program. Levels of 10 copies/3 μL blood or lower for TREC and 6 copies/3 μL blood or lower for KREC at birth are considered to

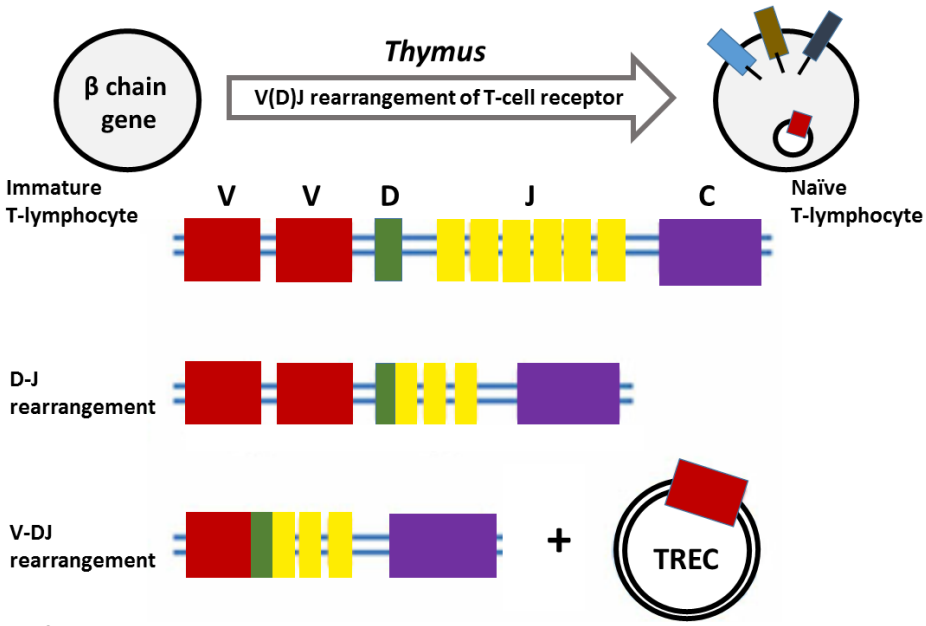

Fig 9.
Exampel of T-lymphocyte receptor gene rearrangement in ß chain, modified according to Lang et al [120].

be below normal values.[122] Severe forms of primary immunodeficiencies display values below cutoff. Further, infants to mothers treated with immunosuppressive medication during pregnancy can show decreased levels at birth, which normalize spontaneously after a few weeks.[122] A wide range of infant's conditions such as preterm delivery, multiple birth, malformations, different genetic disorders, neonatal leukemia, or maternal conditions such as autoimmune diseases, HIV infections, and other maternal medications are associated with low levels of TREC and KREC, even if they are not always below the cutoff.[122, 123] Normal levels differ between several studies, but medians around 200 copies/µL blood (range, < 20 - > 5000) have been reported.[124]

Epigenetic mechanisms, such as histone modification and DNA-methylation play a crucial role in the rearrangement of B-and T-lymphocytes.[125, 126] For example, under the process of antigen receptor gene assembly via V(D)J recombination in B-cells, recent research could present a DNA-demethylation after the first recombination step in the DJ junction.[125] Further, during differentiation of naïve T-lymphocytes, the promoter-enhancer region of the interleukin-2 gene becomes demethylated.[127]

3 Aims

The overall objective of this thesis was to study how mode of delivery and other perinatal factors can affect the epigenetic and immunological state of the newborn infant.

The specific aims were:

- To determine global DNA-methylation in white blood cells (WBC) of the newborn infant in relation to mode of delivery *(Paper I)*.
- To determine global and gene/gene region specific DNA-methylation – involving gene regions relevant for later immune function – in neonatal CD34+ hematopoietic stem/progenitor cells and relating the findings to mode of delivery *(Paper II)*.
- To study contributions from mode of delivery, infant and maternal characteristics to levels of newly formed T- and B-lymphocytes (T-cell receptor excision circles (TRECs) and κ-deleting recombination excision circles (KRECs) *(Paper III)*.

4 Methods

4.1 Study subjects and blood sampling

4.1.1 Paper I

Subjects. All study participants were born at the delivery units at Danderyds Hospital and at Karolinska University Hospital in Stockholm, in total 37 (17 girls) healthy newborn infants at term (gestational age 40 ± 2.6 weeks). Multiple pregnancy, maternal diabetes, hypertension, preeclampsia, medication during the index pregnancy, preterm delivery, neonatal asphyxia, malformations, chromosomal disorders, or congenital infection were all exclusion criteria. The mean maternal age was 33 ± 4.7 years, 11 out of 37 mothers were primigravida, one pregnancy had resulted from in-vitro fertilization and one mother smoked during early pregnancy. All infants had normal birth weights (3699 ± 379 g). Spontaneous VD was the mode of delivery in 21 infants, whereas the other 16 infants were delivered by elective CS under spinal analgesia and before start of labor. Characteristics of the study subjects are shown in *Table 1*.

Table 1. Subject characteristics, n = 37.

	VD	CS
	n = 21	n = 16
Maternal age, years	31.8 ± 3.5	34.5 ± 5.6
Pre-pregnancy BMI, kg/m2	23.0 ± 3.1	24.0 ± 3.0
Parity, n	1.8 ± 0.7	2.0 ± 0.6
Gestational age, weeks	40.7 ± 3.3	38.9 ± 0.5
Birth weight, g	3723 ± 425	3668 ± 321
Girls/boys	9/12	8/8

Data are mean ± SD or proportions. There were no statistically significant differences between the two groups.

Blood sampling. From the umbilical cord, we sampled 5 ml EDTA blood directly after delivery. In conjunction with the metabolic screening test recommended for all infants born in Sweden, 2 ml EDTA blood was also sampled from a peripheral vein at day 3 to 5 of postnatal age.

DNA was extracted and methylation was measured by Luminometric methylation assay (LUMA, section 4.2.1 and 4.2.2).

4.1.2 Paper II

In this study, we recruited pregnant women from the delivery units at Danderyds Hospital in Stockholm, Sweden. Exclusion criteria were multiple pregnancies, maternal diabetes or gestational diabetes, maternal hypertension, preeclampsia, smoking during the index pregnancy, preterm delivery (gestational age < 37 weeks), small-for-gestational-age infants (birthweight ≥ 2 SD below the mean for a Swedish reference population,[128] neonatal asphyxia (Apgar score < 7 at 1 and 5 minutes), malformations, chromosomal disorders, or congenital infection. No pregnancy resulting from assisted reproductive technology was included in the study.

For measurement of global DNA-methylation by LUMA in cord blood stem cells, we included 40 infants (18 girls) to women delivered by elective CS before the start of labor and under spinal analgesia, and as reference group, 49 infants (22 girls) born after spontaneous, non-assisted VD were included. After cell separation and DNA extraction from stem cells (see section 4.2.1), 43 samples (18 CS and 25 VD) contained sufficient DNA (> 500 ng DNA) for methylation analyses.

In the VD group, the start of labor was defined as the time point at which the pregnant woman for the first time perceived regular (3-4 per 10 minutes) and painful uterine contractions. When admitted to the hospital, all women were asked about the time point (hours and minutes) for the start of labor. If labor had not started before admission, the start of labor was noted by the attending midwife. Deliveries with induction of labor were not included in this study.

Indications for CS included previous CS, breech position, pelvic disproportion or maternal request. In the VD group, the median duration of labor was 14.5 hours (range, 1 - 53 hours), and the median duration of ruptured membranes was 4 hours (range, 0 - 17 hours).

Because of insufficient amount of blood samples from the first group, a second group of infants was recruited. Cord blood from 12 infants (6 CS) was used to fill 1 Illumina 450K array (Illumina, San Diego, CA) to measure genome-wide, locus-specific DNA methylation.

A misclassification error occurred regarding gestational age in the clinical records and one preterm infant (GA 33 weeks, CS group) was inadvertently included in the genome-wide, locus-specific DNA-methylation analysis by Ilumina 450K. The characteristics and results from this infant and mother were excluded from all statistical calculations comparing CS with VD. Consequently, 11 samples instead of 12 were analyzed from the Illumina 450K BeadChip.

The DNA content in 9 of these blood samples was sufficient for subsequent validation analysis using bisulfite pyrosequencing. To increase the numbers and power of the validation analysis, we recruited an additional 10 infants (4 CS) to

the second study group. There were no differences in maternal characteristics, gestational age (GA), sex distribution, or birthweight between the first and second study groups.

In the whole cohort of 64 mothers, the median maternal age was 35 years (range, 23 - 43), the body mass index (BMI) was 22.8 kg/m2 (range, 15.9 - 38.4), and 17 of 64 mothers were primigravida. The GA was 39.2 (range, 37.6 - 42) weeks, and all infants had birthweights appropriate for gestational age (3667 g; range, 2820 - 4915 g).

Mothers in the CS group (n = 27) were older compared to mothers in the VD group (n = 37) (37 (range, 25 - 43) vs 34 (range, 23 - 41) years; p = 0.03), and GA was shorter in the CS group compared to the VD group (38.9 (range, 37.7 - 39.9) vs 40.3 (range, 37.6 - 42.0) weeks; p < 0.001). Maternal and infant characteristics by mode of delivery for LUMA, Illumina, and bisulfite pyrosequencing groups are presented in Table *(Paper II)*.

Blood sampling and preparation of hematopoietic stem/progenitor cells. From all participants, 15-20 mL blood was sampled in EDTA tubes from the umbilical cord directly after cord clamping. The cord was clamped after 30 seconds to obtain the targeted volume of cord blood.

Blood cells were sorted and DNA from stem cells was extracted (*Section 4.2.1*).

4.1.3 Paper III

This population-based cohort was based on 7.174 singleton live-born infants, delivered in February through April 2014 in Stockholm county, Sweden. We restricted the study population to infants born at 35 completed weeks of gestation or more, with Apgar scores ≥ 7 at 1, 5 and 10 minutes, and without any record of ICD-10 diagnoses for congenital malformations or neonatal morbidity (the Q- and P-chapters in ICD-10). After these exclusions of 1,160 infants, the study population comprised 6,014 infants and their mothers *(Fig 1, paper III)*.

Using the Stockholm-Gotland Obstetric Data Base, we retrieved maternal, pregnancy, delivery and infant characteristics.[129] Information was prospectively collected; from the first antenatal visit that occurred during the first 13 gestational weeks in 90 % of pregnancies, until postpartum hospital discharge.

The main exposure, mode of delivery was categorized as non-instrumental VD, instrumental VD, emergency or elective CS. Elective CS was defined as CS before onset of labor.

Maternal age was computed from the national identification number (mother's birth date) at the date of delivery. Parity and self-reported smoking habits were based on information obtained at the first antenatal visit. Maternal body mass index (BMI; kg/m2) in early pregnancy was calculated from self-reported

height and measured weight at the first antenatal visit. Maternal diabetes or hypertensive disease was defined as recorded ICD-10 diagnoses: diabetes included pre-gestational (ICD-10 codes E10-E14 and O240-O243) and gestational diabetes (ICD-10 code O244). Hypertensive diseases included pre-gestational (ICD-10 codes I10-I15, O10, O11) and gestational hypertension (ICD-10 code O13), and preeclampsia (ICD-10 codes O14-O15). Gestational age was determined by the following hierarchy: a) date of embryo transfer (2.8 %), b) early second trimester ultrasound (96.1 %), c) date of last menstrual period (1.1 %), and d) from a postnatal assessment (< 0.1 %). Birth weight for gestational age was estimated according to the sex-specific Swedish reference curve for normal fetal growth.[128] A normal birth weight for gestational age was defined as a birth weight between the 3rd and 97th percentiles. Postnatal age at blood sample was calculated by subtracting sampling date and time from birth date and time.

The outcome variables, blood levels of TREC and KREC, were determined at the PKU laboratory at the Karolinska University Hospital, Stockholm, Sweden. TREC- and KREC-levels were analyzed as part of a neonatal screening project to detect severe combined immune deficiencies.[122] Perinatal and maternal covariates were categorized as presented in Table 2 (*section Results*) and *S1 Table, paper III*.

4.2 DNA methylation analyses

4.2.1. DNA extraction and cell sorting

DNA was extracted using commercially available reagents (Nucleon BACC3 kit, GE Healthcare Europe GmbH, Freiburg, Germany). DNA quantification was performed by using the NanoDrop ND-1000 (NanoDrop Technologies Inc./Thermo Fisher Scientific Inc., Wilmington, DE, USA) (*Paper I and II*).

In *paper II* we sorted umbilical cord blood cells with commercially available tools (Dynabeads positive isolation kit; Invitrogen by Life Technologies Corp, Carlsbad, CA) to separate CD34+ stem cells from other DNA-containing cells.

CD34 (cluster of differentiation 34) is a glycosylated transmembrane protein[130] and can be used for cell isolation, i.e. immunomagnetic methods as described above. CD34 is expressed and represents a well-known marker for hematopoietic stem cells but also for early stages of progenitor cells,[131] the first step of hematopoietic differentiation *(Fig 8).* Although we consistently applied the term hematopoietic stem cell or CD34+ hematopoietic stem cell in *paper II,* we are aware of that some of them already represent progenitor cells.

The isolated DNA were then frozen at -20°C until analysis by LUMA, Bisulfite pyrosequencing or Illumina 450k methylation bead array. All DNA

methylation analyses were performed with the investigators blinded to mode of delivery.

There are two main technologies available to measure DNA-methylation. In the first method, total global DNA methylation is quantified by making use of methylation-sensitive restriction enzymes or immunoprecipitation with an antibody against methylated cytosine.[132] LUMA belongs to this group. The second method applies bisulfite-based treatment to convert unmethylated cytosine to uracil followed by sequencing or array technologies.[132] Bisulfite pyrosequencing and Illumina 450k are based on the second technology.

4.2.2 LUMA (Luminometric methylation Assay) (Papers I and II)

LUMA was used for examining the global DNA-methylation. The basis of this method is DNA cleavage by methylation-sensitive or -insensitive restriction enzymes followed by a bioluminometric polymerase extension assay to quantify the degree of restriction cleavage.[133] Isoschizomer HpaII and MspI (restriction enzymes with the same recognition sequence) endonucleases are used. Both enzymes have the recognition sequence CCGG. However, HpaII cleavage is inhibited if the internal cytosine in the DNA is methylated (CmCGG), while MspI always cuts the recognition sequence, regardless of methylation status.[134]

The DNA, treated with the enzymes leaving an extendable 5'-overhang, was then analyzed by a luminometric polymerase extension assay to quantify the amount of restriction cleavage by each of the enzymes. The relative amount of DNA methylation was defined as the HpaII/MspI ratio. The HpaII/MspI ratio would be 1 (equal amount of restriction) when DNA was completely unmethylated, and would approach 0 when DNA was fully methylated. Parallel reactions, one with HpaII and one with MspI, were run. To enable normalization between runs and for DNA input, EcoRI was included in all reactions. EcoRI has the recognition sequence GAATTC, and this cleavage was not affected by CpG methylation. After HpaII or MspI restriction at their recognition sequence, there was a resulting 5′-CG overhang, whereas EcoRI restriction produced a 5′-AATT overhang. Using the Pyrosequencing® platform, nucleotides were sequentially added *(Fig 10)*.

The entire LUMA analysis process was described in Methods in molecular biology.[134]

The digestion reactions were run in a 96-well format using a PSQ96TM MA system (Biotage AB, Uppsala, Sweden). Peak luminometric heights were calculated using the PSQ96TM MA software.

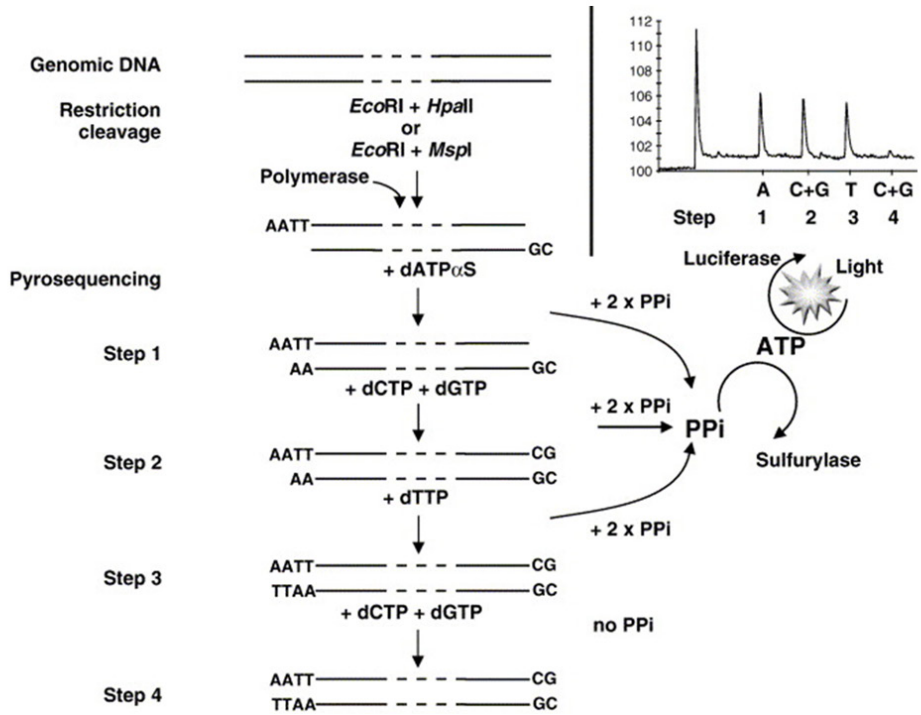

Fig 10.
"Analysis of global DNA methylation with the LUMA assay. Genomic DNA of the test sample is cleaved with the two combinations of restriction enzymes, either HpaII + EcoRI or MspI + EcoRI. The extent of cleavage is then determined by a polymerase extension assay based on a four-step pyrosequencing reaction. After each nucleotide dispensation, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase. Luciferin is converted by Luciferase and ATP to produce a proportional amount of visible light that is detected by a CCD (Charge-coupled device) camera. The amount of light is directly proportional to the number of overhangs produced by the respective restriction enzymes. The A and T peaks correspond to pyrosequencing Step 1 and Step 3, reflecting the EcoRI cleavage and should be equal. The C + G peak resulting from pyrosequencing Step 2 illustrates HpaII or MspI cleavage. The second C + G peak originating from Step 4 is an internal control that should be close to zero." According to Karimi et al.[133]

4.2.3 Bisulfite pyrosequencing analysis (Paper II)

Bisulfite pyrosequencing is based on DNA treatment with bisulfite which converts cytosine to uracil, but leaves methylated cytosine unaffected *(Fig 11)*.

Pyrosequencing (or regular sequencing) is thereafter applied to determine the bisulfite-converted DNA sequence, where specific CpG sites in the sequenced regions can be assessed.[135]

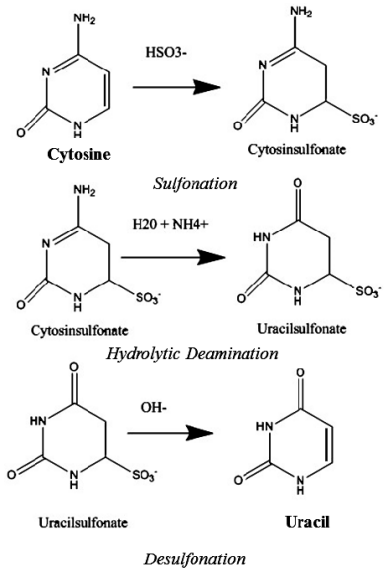

Fig 11.
Conversion of cytosine to uracil, modified according to Tollefsbol(ed), Handbook of Epigenetics 2011.

We selected CpGs, associated with the genes *COLEC11*, *PCK2, PGBD5*, and *HLA-F*. These genes had possible clinical relevance and/or CpGs exhibited the largest DNA methylation differences in relation to the mode of delivery in the genome wide analysis by Illumina 450k *(see next section)*.

Genomic DNA (500 ng) was treated with sodium bisulfite (EpiTect bisulfite kit; QIAGEN, Valencia CA). One microliter of converted DNA (~10 ng) was applied as a template in the PCRs performed with the PyroMark PCR kit (QIAGEN). The entire PCR product and 4 pmol of the respective sequencing primer, and streptavidin sepharose high-performance beads (GE Healthcare), were used for pyrosequencing performed with the PSQ 96 system and the PyroMark Gold Q96 reagent kit (QIAGEN). The PyroMark CpG software 1.0.11 served for data analysis.

4.2.4 Genome-wide, locus-specific DNA-methylation analysis (Paper II)

For the genome-wide and locus-specific DNA-methylation, we used the Illumina Infinium HumanMethylation450 BeadChip (450K), a cytosine microarray, based on the Infinium Technology.[135] It is a quantitative DNA methylation measurement

of bisulfite-treated genomic DNA. Illumina 450K contains more than 480,000 probes, targeting 99 % of genes and 96 % of CpG island regions.[135] Illumina 450K can detect differences of 10 % or more in methylation with 99 % confidence.[135]

One chip was used, allowing for 12 samples (6 CS and 6 VD) from cord blood CD34+ cells to be analyzed. For each CpG and sample, the methylation level was estimated as a ratio (β) of the methylated signal to the sum of methylated and unmethylated signals. Before normalization, CpG sites located at known single-nucleotide polymorphisms (list provided by the manufacturer) were discarded to avoid potential confounding by single-nucleotide polymorphisms at CpGs as well as probes located on the X and Y chromosomes. In addition, we discarded CpG probes with detection values of $p > 0.01$.[136]

To estimate the β values for the included CpG probes, a 3-step pipeline, described as optimal, was used.[132] Differential DNA methylation was computed by transforming β values into M values[137] and using a software package for the analysis of gene expression microarray data, limma (Linear Models for Microarray Data), to define a linear model. Differentially methylated positions (DMPs) were defined as those that exhibited a 10 % or greater difference in the DNA methylation between the DMPs of the VD and CS groups, at a value of $p < 0.01$.

We chose the definition above to expose the most important DMPs, considering the following:

(1) most differences in methylation are small

(2) we investigated a lot of probes that required multiple testing

(3) we had only a small number of samples.

We regarded a 10 % difference of DNA-methylation as a conservative doorstep for a greater difference in DNA methylation between CS and VD. We used the annotation from Illumina, and summarized the DMP information over genes by computing the number of probes for each gene, the number of DMPs, and how many DMPs were hyper- or hypomethylated.

4.3 TREC/KREC analyses (Paper III)

The original dried blood spots for the national neonatal screening program were used to determine TREC and KREC levels in 3.2 mm punches. DNA from a single 3.2-mm punch of the dried blood disks was eluted into 24 μL of Generation DNA Elution Solution (QIAGEN) supplemented with 100 μg/mL yeast tRNA (Ambion), and 8 μL was subjected to real-time quantitative PCR (RT-qPCR) of TRECs, KRECs, and β-actin (ACTB) in a 96-well format.[138, 139] TREC and KREC copy numbers were normalized per microliter of blood, assuming, that a 3.2-mm punch contains about 3 μL of whole blood. ACTB amplification was used to assess the success of DNA extraction from the Guthrie cards. A cycle threshold for

TREC, KREC, or ACTB was fixed for automated data collection and analysis of the amplification during the exponential phase. By 10-fold serial dilution using a TREC-KREC-TRAC (TCRα subunit constant gene) construct containing plasmid and a β-actin sequence containing plasmid, Calibration curves were generated.[139]

4.4 Statistical analyses

4.4.1 Paper I and II

Data were expressed as mean values and standard deviation (normally distributed variables) or median values and range (skewed distribution). Statistical analysis was performed using rank sum tests (Mann-Whitney U-test and Wilcoxon signed-rank test) and associations were tested for by calculating Spearman´s correlation coefficients. A p-value < 0.05 was considered statistically significant.

All analyses regarding the locus-specific methylation were performed using the R statistics software.

The power calculation for the sample size in paper II was based on findings in paper I, in which DNA methylation (HpaII/MspI ratio) in the VD and CS groups had the mean values (SD): 0.30 (0.046) and 0.25 (0.022). Assuming normal distribution and equal variance, the power calculation estimated 10 samples in each of the 2 groups would be needed to detect a difference in the DNA methylation of the same magnitude (2-sided test, 5 % significance level, 80 % power). The power calculation was performed in STPLAN version 4.5 (Obtained from MD Anderson Cancer Center. Available at: https://biostatistics.mdanderson.org/SoftwareDownload/SingleSoftware.aspx?Software_Id=41. Accessed June 12, 2014).

Spearman´s correlation was used to compute the correlation between duration of labor and β value of each probe. Kruskal Wallis test was used to compute the significance of the difference between DMPs vs non-DMPs. Considering the all-probe correlations as a null distribution, we used as significance thresholds the 0.01 and 0.99 quantiles to identify significant correlations in the DMP set *(Fig 15, section Results 5.3).*

4.4.2 Paper III

Perinatal characteristics were described as numbers and rates, and compared by chi-squared test. Levels of TREC and KREC were log-transformed to achieve a normal distribution, and divided into quintiles, from low to high values. Chi-square test was used to estimate differences in distributions of exposure (mode of delivery and other perinatal covariates) in relation to TREC- and KREC-quintiles. In addition, linear regression models were used to investigate whether the log-transformed TREC- or KREC-values were associated with our perinatal

covariates. We also used logistic regression to calculate odds ratios with 95 % confidence intervals for the risk of having a TREC- or KREC-value within the lowest quintile (Q1), with the other quintiles (Q2-Q5) as the reference category. As postnatal age for blood sampling was strongly associated with TREC- and KREC-values, we investigated whether postnatal age for sampling modified associations. A statistical interaction was pre-defined as an interaction term with p-value < 0.05. Finally, sensitivity analyses were performed in the subset of infants with mothers without recorded diabetes and hypertensive disease.

The SAS software package version 9.4 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

4.5 Ethical approvals

The studies were approved by the regional ethical vetting board at Karolinska Institutet in Stockholm (Paper I, Dnr 2007/37-31/3) and by the Regional Ethical Review Board in Stockholm, Sweden (Paper II and III, Dnr 2010/440-31/4; 2012/1029/32, 2014/1292-31/4).

5 Results

5.1 Global DNA-methylation in white blood cells after Cesarean section (Paper I)

At birth, global DNA-methylation was significantly higher in infants delivered by elective CS as compared to those born by normal VD ($p < 0.001$). Three to five days after birth, the difference in global DNA-methylation between the two groups was smaller and did no longer reach statistical significance ($p = 0.10$). In vaginally delivered infants, global DNA-methylation did not change between birth and 3-5 days of postnatal age ($p = 0.55$). However, in infants delivered by CS, DNA-methylation decreased significantly over the same period of time ($p = 0.01$) *(Table 2, paper I and Fig 12).*

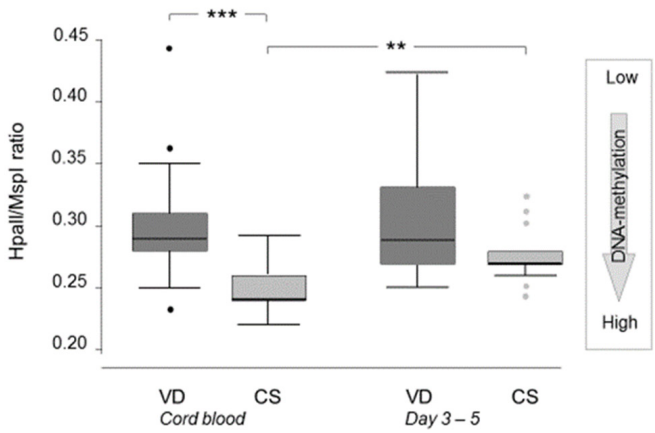

Fig 12. Mode of delivery and global DNA-methylation in healthy newborn infants. DNA-methylation in white blood cells expressed as HpaII/MspI-ratio.

Neonatal DNA-methylation did not correlate to maternal risk factors (age, pre-pregnancy BMI, parity, maternal folate, and CRP-levels), perinatal risk factors (gestational age, duration of delivery, duration of ruptured membranes), or infant risk factors (sex, birth weight, folate, and CRP-levels) (p-values varying between 0.16 – 0.98). Accordingly, no multivariate analyses were performed.

5.2 Mode of delivery and global DNA-methylation in hematopoietic/progenitor stem cells (Paper II)

CD34+ hematopoietic stem cells presented significantly more methylated DNA - determined by LUMA - in cells from CS infants compared to cells from VD infants *(p = 0.02, Fig 13)*.

Global DNA methylation in the neonatal CD 34+ stem cells did not correlate with maternal characteristics (age, pre-pregnancy BMI, parity, duration of delivery, and duration of ruptured membranes) or infant risk factors (GA, sex, birthweight) (p-values varying from p = 0.13 to p = 0.91).

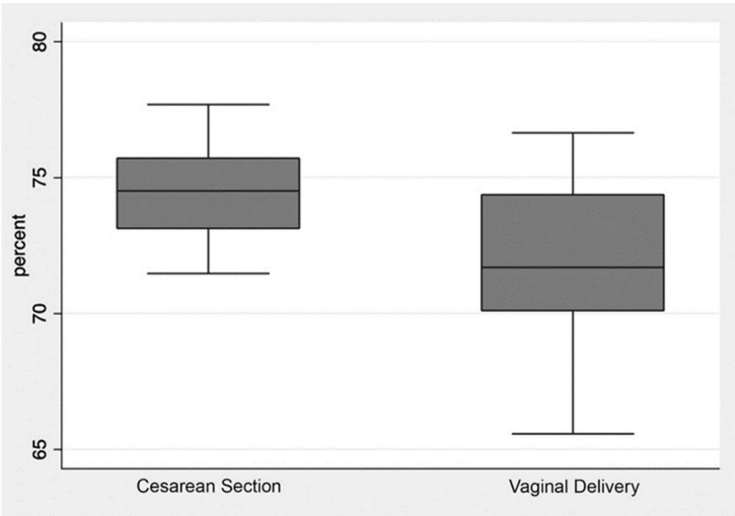

Fig 13. Global DNA methylation of CD34+ cells in cord blood was significantly higher in the group of infants born after elective CS compared to infants born after VD (p = 0.02).

5.3 Mode of delivery and genome-wide, locus-specific DNA-methylation in hematopoietic stem/progenitor cells (Paper II)

We assessed locus-specific DNA methylation to detect specific genes differentially modified in relation to mode of delivery. This resulted in the identification of 343 DMPs exhibiting a difference in DNA methylation of 10 % or more *(p < 0.01, Fig 14 and Suppl Table 2)*. The maximal locus-specific difference in DNA methylation in CD34+ stem cells from CS and VD infants was 40 %. Among the 343 DMPs, there were 179 (52 %) associated with known genes and a majority of the DMPs were found in gene bodies.

In contrast to the results from the global DNA-methylation analysis of hematopoietic stem cells (chapter 5.2), a majority (260 of 343, 76 %) of the DMPs in the neonatal CD34+ stem cells were found to be hypomethylated in CS as compared to VD *(Fig 14)*.

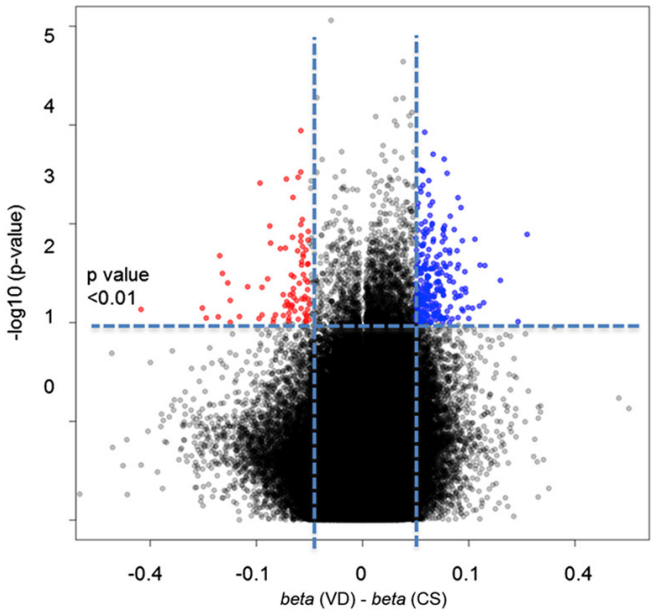

Fig 14.
A volcano plot of log-transformed p values vs differences in DNA methylation (b-value) between CS and VD. Horizontal and vertical lines denote thresholds for the definition of differentially methylated positions > 10 % difference in methylation, p < 0.01). Red dots indicate hypomethylated, blue dots indicate hypermethylated in VD vs CS.

We found also a strong CpG-specific relationship between the duration of labor and locus-specific DNA-methylation in a subset of three identified genes (*IRS1* (r = 0.91/p = 0.01), *PRDX1* (r = 0.93/p = 0.01), and *SORT1* (r = 0.86/p = 0.02), in addition to a general trend of higher correlation between duration of labor and DMPs *(Fig 15)*.

Fig 15 shows the distribution of the correlations; for all non-discarded probes, the distribution is centered around 0 (mean, −0.05; median, −0.04), whereas for DMPs, the correlation is larger (mean, 0.1; median, 0.17). The significance of the difference between DMPs vs non-DMPs computed by a Kruskal Wallis test was p < 0.001.

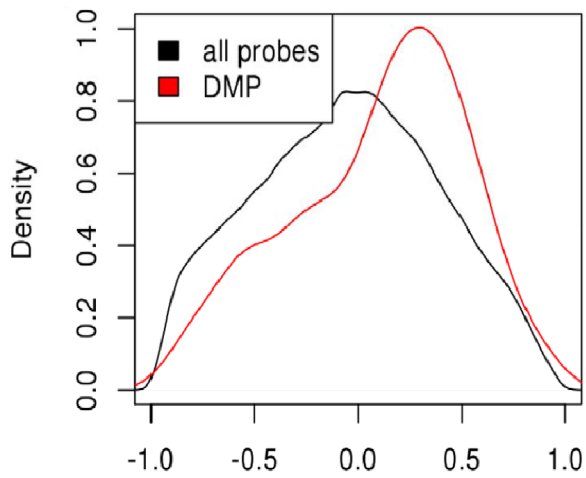

Fig 15.
The black line denotes the density of correlation between VD-methylation and duration of labor for all CpGs (null distribution), and the red line denotes it for all DMPs. The right-shifted distribution for DMPs (red line) indicates hypermethylation. X-axis denotes the correlation; y axis denotes the kernel density estimate as computed by density function in R.

In these selected gene-associated probes, the degree of locus-specific DNA methylation in CS was similar to that in VD infants exposed to a short duration of labor, whereas at longer duration of labor in the VD group, the differences gradually increased *(Fig 3, paper II)*.

For validation analysis, we replicated by bisulfite pyrosequencing eight CpGs associated with the genes *COLEC11*, *PCK2*, *PGBD5*, and *HLA-F*. The CpGs displayed the largest methylation differences by Illumina 450K in relation to mode of delivery and some of the genes may have relevance to processes in the immune system. From previous research are following facts about their function and importance known:

- *COLEC11* gene encodes a member of the collectin family of C-type lectins that possess collagen-like sequences and carbohydrate recognition domains. Collectins are secreted proteins that play important roles in the innate immune system.[140]
- *PCK2* gene encodes an isozyme of phosphoenolpyruvate carboxykinase, the phosphoenolpyruvate carboxykinase 2 (PEPCK-M). PEPCK-M is the first committed step of gluconeogenesis and glyceroneogenesis in the liver.[141]
- *PGBD5*, the piggyBac transposable element derived 5, gene encodes an active DNA transposase. The transposase is expressed in the majority of childhood solid tumors, including lethal rhabdoid tumors.[142]
- *HLA-F* (major histocompatibility complex, class I, F) gene encodes a HLA class I heavy chain paralogue. The function is still not clear but it is supposed to bind a restricted subset of peptides for immune presentation and to activate cytotoxic T-lymphocytes.[102] This gene has also been associated with a genetic predisposition to type 1 diabetes.[143]

The direction of methylation differences by bisulfite pyrosequencing in CS vs VD infants corresponded to the methylation analysis by Illumina 450K *(Fig 4, paper II)*. The sites of the DMPs for these genes varied with respect to the genes, including locations in both intragenic and 5′-untranslated region.

The differentially methylated CpG associated with *COLEC11*, was located 10 bases upstream of exon 1. Two of the *PCK2*-associated CpGs were located in the 5′-untranslated region 180 and 185 bases upstream the transcription start site. The other 2 CpGs were located intragenic between exons 1 and 2. The differentially methylated CpGs associated with *PGBD5* had an intragenic location between exons 2 and 3, and the CpG in *HLA-F* was intragenic between exons 1 and 2. For the exact chromosomal location, see Suppl. Table 1, paper II.

The GREAT (Genomic Regions Enrichment of Annotations Tool), developed by Bejerano et al, is a web-based tool to associate biological functions to genomic

regions.[144] The particular purpose of this analysis is to allocate biological meaning to non-coding genomic regions by analyzing the annotations of the nearby genes.[144] Three significant molecular functional annotations were revealed when analyzing our data: phosphoenolpyruvate carboxykinase activity, ligand-regulated transcription factor activity, and mouse double minute-2 binding. Furthermore, GREAT associated our gene set to more than 20 biological processes. The top 4 were: (1) immunoglobulin biosynthetic process, (2) regulation of glycolysis, (3) regulation of cellular ketone metabolic process, and (4) regulation of response to food *(Fig 16)*. Two signaling pathways into cells were identified as differentially methylated: the Cadherin signaling and the Wnt signaling pathways.

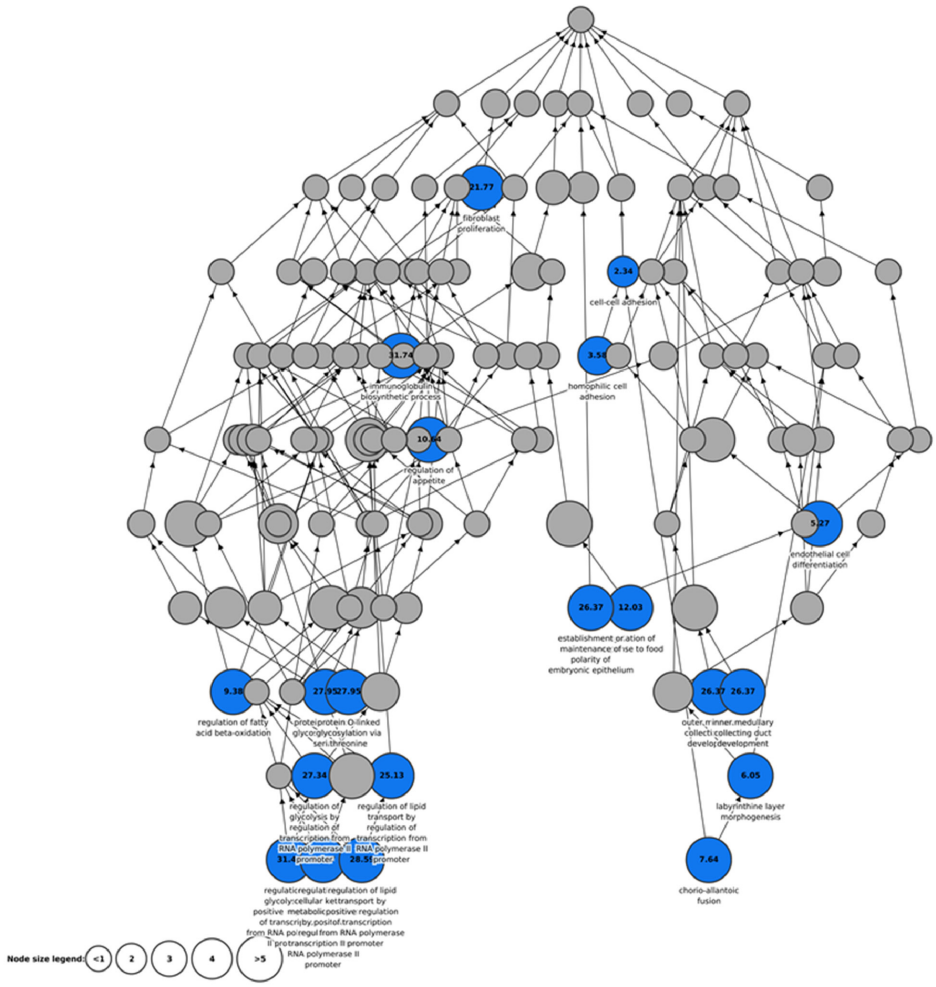

Fig 16.
Gene ontology term, biological process, identification by GREAT.
A directed acyclic graph structure of the gene ontology hierarchy displays enriched nodes based on DMPs. Each colored node represents a significant biological process and the size of the node corresponds to the number of associated DMPs.

5.4 Immune cell formation at birth, mode of delivery and infant characteristics (Paper III)

Neonatal blood levels of TREC ranged from 16 - 622 copies (per 3.2 mm punch dry blood spot) with a median value of 166 copies. KREC blood levels ranged from 2 - 638 copies/3.2 mm punch, with a median of 97 copies.

In the study population of 6,014 singleton infants born between 35 and 42 gestational weeks, low TREC- and KREC-levels (within the first quintile) were more commonly found after elective CS, in male infants, after preterm birth (35 - 36 week), in infants with low birth weight for gestational age, and when the blood sample was taken within the first 3 - 4 postnatal days *(Table 2)*. In addition, the distributions of TREC-levels were left-shifted (towards lower levels) in infants delivered by elective CS compared to those delivered vaginally *(Fig 17)*, in male compared to female infants, after preterm (35 - 36 weeks) compared to term birth (37 - 41 weeks), and in infants with low birth weight for gestational age as compared to infants with appropriate birth weight for gestational age *(Fig 3 - 5, paper III)*. In contrast, rates of low TREC and KREC did not differ by any maternal characteristics, such as age, parity, BMI and smoking *(S1 Table, paper III)*. However, the rate of a low TREC-level was higher in infants of diabetic mothers, and the rate of a low KREC-level was higher in infants of mothers with hypertensive disease.

The proportions of infants with TREC- and KREC-levels in the lowest quintile decreased with infant postnatal age and therefore, all logistic regression models were adjusted for postnatal age at blood sampling.

In the adjusted models, elective CS, male infant sex, preterm birth at 35 to 36 weeks' gestation and smallness for gestational age (SGA) were associated with increased odds for a TREC-level in the lowest quintile *(Table 2, paper III)*. Male infants, infants born post-term (≥ 42 weeks of gestation) and SGA-infants had increased odds for low KREC-levels, whereas elective CS was not associated with increased risk for low KREC *(Table 2, paper III)*. Maternal characteristics showed no consistent associations with risks of low TREC or KREC levels (S2 Table, paper III). As mode of delivery may be related to gestational age, we tested whether gestational age modified associations between mode of delivery and low TREC and low KREC, respectively. However, gestational age did not interact with mode of delivery (interaction terms $p = 0.29$ and $p = 0.97$, respectively).

Given the strong and inverse association between postnatal age and low TREC- and KREC-levels, we investigated whether postnatal day for blood sampling modified the associations between other perinatal covariates with regard to risks of low TREC- or KREC-levels. However, we found no statistical interactions between postnatal day for blood sampling and mode of delivery, gestational age,

infant sex or birth weight for gestational age, respectively.

Since rates of low TREC- and KREC-values were higher in infants to mothers with diabetes and hypertensive disease *(S1 Table, paper III)*, we repeated the logistic regression analyses within the subset of 5,681 infants to mothers without diabetes and hypertensive disease. Compared to the associations within the complete cohort, similar results were found in these sub-analyses *(S3 Table, paper III)*.

		Low TREC			***Low KREC***		
	Total nb	***nb***	***rate (%)***	*p-value**	***nb***	***rate (%)***	*p-value**
Mode of delivery							
Elective C-section	640	176	(27.5)	*<0.01*	156	(24.4)	*0.02*
Emergency C-section	452	100	(22.1)		96	(21.2)	
Instrumental vaginal	325	58	(17.9)		64	(19.7)	
Non-instrumental vaginal	4597	869	(18.9)		887	(19.3)	
Infant sex							
Male	3068	728	(23.7)	*<0.01*	680	(22.2)	*<0.01*
Female	2946	475	(16.1)		523	(17.8)	
Gestational age							
35-36	96	34	(35.2)	*<0.01*	23	(24.0)	*<0.01*
37-41	5457	1078	(19.8)		1061	(19.4)	
42	461	91	(19.7)		119	(25.8)	
Birth weight for gestational age							
SGA, <3 perc	74	25	(33.8)	*0.03*	36	(48.7)	*<0.01*
AGA, 3-97 perc	5801	1153	(20.0)		1156	(20.0)	
LGA, >97 perc	137	24	(17.5)		11	(8.0)	
Missing	2	-	-		-	-	
Postnatal age at blood sample							
2	3147	809	(25.7)	*<0.01*	894	(28.4)	*<0.01*
3	1800	281	(15.6)		227	(12.6)	
4-10	1	058	112	(10.6)	82	(7.8)	
Missing	9	1	-		1	-	

** According to chi square-test.*

Table 2.
Perinatal characteristics of 6,014 singleton infants born at 35-42 weeks of gestation, and numbers (nb) and rates of TREC- and KREC-levels in the lowest quintile.

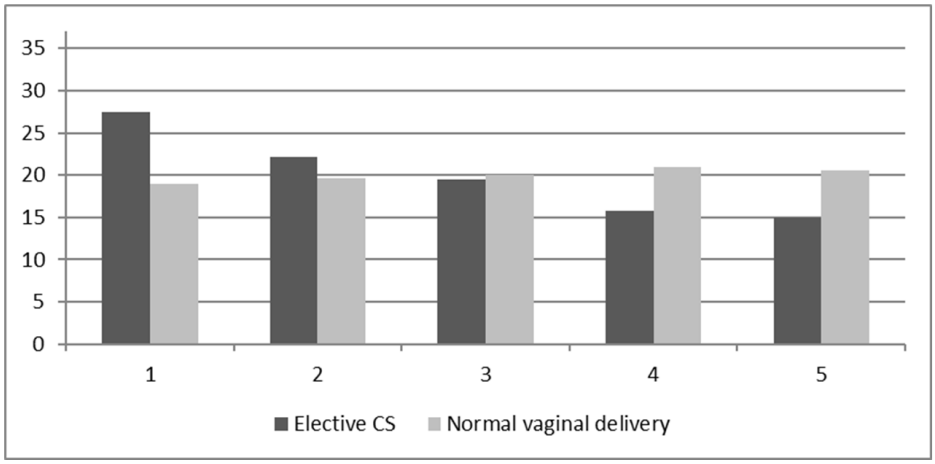

Fig 17.
Rates (%) of TREC values in quintiles (1=lowest quintile) after elective CS and normal non-instrumental VD, respectively. (Chi-square p-value < 0.001)

6 Discussion

6.1 Finding and implications

Mode of delivery and DNA-methylation, an epigenetic mechanism

Accumulating evidence suggests that conditions, early in human life, i.e. in utero, during and immediately after birth, may affect future health.[145, 146] The mechanisms for such early imprints are presumptive epigenetic and may remain for many years.[147] Genes may be equilibrated by these early epigenetic modifications for a response to future triggers, like infections, toxicants, aging processes etc. Accordingly, the frames for cellular and organ functions may be determined long before they are challenged.[3] The increasing rates of CS, the conditions of birth stress during normal delivery, and the uncertainty about their long-term health consequences requested us to study epigenetic effects related to mode of delivery.

Our novel findings were:

- At birth, global DNA-methylation in white blood cells in cord blood was significantly higher in infants delivered by elective CS as compared to those born by normal VD *(Paper I).*
- Global DNA-methylation in neonatal CD 34+ stem/progenitor cells in cord blood was significantly higher in infants delivered by elective CS as compared to those born by normal VD *(Paper II).*
- Genome-wide methylation analysis identified 343 CpG positions (179 associated with known genes) differentially methylated in cord blood CD 34+ hematopoietic stem/progenitor cells from infants delivered by elective CS, compared to those born by VD. The maximal locus-specific difference was 40 percent.
- In these neonatal CD34+ cells, there was evidence for a relationship between the duration of labor and the degree of DNA-methylation in three specific genes in offspring.
- The GREAT analysis revealed differential methylation in genes and associated biological processes regarding immunoglobulin synthesis, metabolism and apoptosis.

Mode of delivery affects the epigenetic state of the newborn infant is consequently a possible interpretation. Moreover, the GREAT analysis indicated a functional relevance.

The long-term risk for diseases associated with delivery by CS is of great importance. CS rates are still accelerating and in many parts of the world is it a very common delivery mode, in some parts the most common.[30, 40]

The stress of being born vaginally is suggested to be of importance for physiological transition and survival of the fetus leaving the womb and entering the outside world.[12, 13] Infants born by elective CS before onset of labor desiderate this form of preparation and may therefore be maladaptive for those infants. In addition to the more obvious disadvantages for circulation and respiration, also activation of the immune system seems to be influenced. Previous studies have identified differences in immune biomarkers related to CS compared to VD, and even experimental findings suggest that mode of delivery can alter gene expression with a functional significance for the immune system.[19, 40, 67, 148, 149]

The hygiene hypothesis assumes that the missing colonization of the infant with maternal vaginal and gut flora after CS may inhibit the initiation of the immune system of the newborn[67] and the altered neonatal gut colonization leads to an increased risk, for example, to allergic diseases after prelabor CS.[70] We found a correlation between the duration of labor and DNA methylation, indicating that methylation status at the start of vaginal birth is similar to that of CS infants (unexposed to labor and ruptured membranes), after which DNA methylation gradually changes along with duration of delivery. In addition, we found no association between duration of ruptured membranes and DNA-methylation. Thus, it suggests that our findings are linked to labor itself rather than to differences in bacterial colonization.

However, altered gut colonization of the newborn infant may act through other epigenetic modification than studied by us. For example, the immunoregulatory effects of the gut microbiota may be mediated via histone modification in the promoter of the *Foxp3* gene, which enhances extrathymic induction of T-cells.[150, 151] Previous studies indicate that maternal microbial transfer to the offspring may begin during pregnancy, providing a "pioneer microbiome".[152-154] This early life environment of the intrauterine "pioneer microbiome" may imprint the offspring microbiota in preparation for the much larger amount of microorganisms during VD.[152] Thus, mode of delivery might affect the epigenetic state of the newborn infant may act both through maladaptive perinatal stress and altered microbial colonization of the neonatal gut. One central point in this context is that altered methylation associated with CS may create replication-heritable epigenetic marks, not immediately influencing gene transcription until an event later in life occurs and causes disease.

A growing number of recently published studies regarding DNA-methylation in human umbilical cord WBCs support our findings that environmental

factors in early life may affect the epigenetic state. Maternal depression changed DNA-methylation in T-lymphocytes from the umbilical cord of neonates.[155] Maternal stress might also be associated with DNA-methylation changes at the gene sequence for the *MEST* gene, in umbilical cord WBC from newborn infants.[156] Idiopathic preterm delivery influenced the genome-wide, locus-specific DNA-methylation (by the Illumina 450K platform) at between 161 to 196 DMPs, and a number of them was still differentially methylated at 18 years of age.[157, 158] Growth restricted neonates had different DNA-methylation profiles, totally 839 DMPs and 56 DMPs > 10 % (β values by Illumina 450K) compared to AGA infants in cord blood at birth.[159] Prenatal physical activity of mothers was associated with reduced birth weight and DNA-methylation differences of a certain gene, *PLAGL1,* in cord blood samples.[160]

DNA-methylation differences were also found in umbilical cord blood at birth in children with adipositas[161] and ADHD symptoms[162] at six years of age.

After our first published study *(Paper I)*, two studies from other groups performed analyses of global and single gene DNA-methylation and mode of delivery.[163, 164] Virani et al[163] investigated global DNA-methylation in cord blood from elective CS and VD infants using both LUMA and LINE1 analysis. Their cohort was substantially larger than ours, and they did not find differential global DNA-methylation after adjusting for a number of confounders, including smoking. Franz et al[164] used ELISA based on capture of methylcytosine and detection antibodies for global analysis. 96 single genes were analyzed by quantitative PCR using the Methyl-Profiler DNA Methylation PCR Array System. Their cohort was of the same range as ours and they did not find differential global DNA-methylation, but in two of 96 genes, *ELA2* and the *IRF1*, was the DNA-methylation significantly higher in the CS group compared to the vaginally born. It is important to note, however, that the LUMA method or other global analyses give the average methylation levels in the interrogated restriction sites throughout the genome and therefore will display only small changes if there are similar amounts of hyper- and hypomethylation, even if the changes themselves could be substantial. Although, we analyzed only global DNA-methylation in CD34+ hematopoietic stem/progenitor cells, we found yet a small, significant effect of mode of delivery on it. More importantly, using Illumina 450K, larger and bidirectional differences in DNA-methylation were found at hundreds of specific CpG sites in infants born by CS as compared to VD. The finding of significant different methylation of two single genes by Franz et al is in line with our locus-specific results by Illumina 450K.[165] Methylation differences in various cell types in whole blood[166] as well as the different methylation analysis methods may contribute to divergent results from Virani et al. and Franz et al.[163, 164]

Our findings in study I and II have no direct clinical implications today. Given the growing evidence about CS, short- and long-term consequences, the current Swedish guidelines regarding elective CS from 2011 should not be changed.[167]

A different epigenetic profile after CS may be a concernment for umbilical cord blood banks, with intention to transplant cord blood stem cells. Numbers of cord blood cells are influenced by mode of delivery.[168] It is still unknown if delivery mode also could affect transplantation outcomes.

Perinatal characteristics and lymphocytes at birth

Our third study found an association between mode of delivery, other perinatal characteristics and the number of newly formed, naïve T- and B-lymphocytes, determined by measurement of TREC and KREC. To our best knowledge, these associations have not been reported.

Our novel findings in paper III were:

- Delivery by elective CS was associated with a 32 % higher risk of having low numbers of newly formed T-lymphocytes (neonatal TREC values within the lowest quintile) as compared to VD.
- Male sex, delivery at 35 to 36 gestational weeks, and being born SGA were associated with a shift towards lower TREC values (Lowest quintile).
- Male sex, infants born SGA or post-term (≥ 42 weeks of gestation) were also at increased risk of lower levels of B-lymphocytes (KREC values within the lowest quintile).

Other associations between KREC values and mode of delivery and remaining infant characteristics were absent. Moreover, we found no association between maternal characteristics or complications during pregnancy and high or low levels of TRECs or KRECs in the offspring.

Several previous studies reported associations between being born and an immediate leukocyte release.[169-173] Labor and the stress of being born is thought to be a major mediator in the establishment of immune function. Elevated levels of IL-8, soluble E-Selectin and Interferon-c levels were associated with progressive increasing fetal stress during VD.[19, 174] The stress of being born depends on mode of delivery, with much lower sympatho-adrenal activation and lower cortisol levels after elective CS than after VD.[12, 14, 17] For that reason, mode of delivery seems to be an important factor for the postnatal adaption of the offspring immune system.[19]

The findings of our present study extend the knowledge that mode of delivery can affect the adaptive immune system by means of lower levels thymic differentiated T-lymphocytes. If the lower account of naïve lymphocytes is just a transitory neonatal phenomenon remains to be established.

The process of differentiation of T- and B-cells generates a pool of long-lived lymphocytes.[175] If this pool is reduced already from start, it may have clinical consequences in later life, i.e. when the individuals as child or adult become exposed to infectious organisms or develop immunologic conditions like immunodeficiency and autoimmunity. Growing evidence links early fetal-neonatal living conditions and events to health in childhood and adult life.[4] As described, CS is associated with a moderately increased risk for immune disorders later in life.[47] Our results indicate that a reduced number of naïve T-cells after CS could be one possible link. We have not yet evidence and can only speculate if epigenetic mechanisms may be the basis for our findings. What we know is that DNA-methylation plays a role in the differentiation and rearrangement process of T-cells.[125-127]

Besides mode of delivery, male sex was associated with risk for low TRECs and KRECs after delivery. Cord blood from female infants has been found to contain higher levels of CD4+ T-lymphocytes, higher CD4/CD8 T-lymphocyte ratios and lower levels of CD8+ T-lymphocytes and NK cells, compared to cord blood from male infants, whereas the levels of B- lymphocytes is comparable in males and females.[176, 177] Sex differences related to immune disorders have been described.[178] Diabetes type 1,[179] asthma,[180, 181] hypertensive disease,[181, 182] and inflammatory bowel disease[183] affect males differently, and mostly more often, than females. Whereas women are more often affected by rheumatoid arthritis[184] and multiple sclerosis.[185] A disadvantage for male in immune responses to infectious diseases[186] and outcome after sepsis[187] has also been described.

Preterm babies are more vulnerable to infections and have functional deficiencies in their immune system.[188] The influence of immaturity to the overall risk of infection during the neonatal period is not completely understood, but prematurity is regarded to be the major risk factor for infections during this early period of life.[189] In paper III we found that also near-term birth, i.e., at 35-36 weeks, was associated with lower numbers of naïve T-lymphocytes. The analysis displayed also an association between smallness for gestational age and low TREC and KREC levels, respectively. From previous studies, it is known that infants born small for gestational age have a significantly lower number of T- and B-lymphocytes[190] and constitutional small for gestational age fetuses had a disproportionately smaller thymus.[191] The findings implicate that gestational age and fetal growth should be considered when exploring associations between mode of delivery and the neonatal immune system, as well as associations between mode of delivery and later health-related outcomes. Additionally, low birth weight has been identified as a risk factor for adult diseases where inflammation plays a key role, namely hypertension, coronary heart disease, dyslipidemia, type 2 diabetes and obesity.[192-195]

Besides, we also found that TREC and KREC levels altered over time from birth

and therefore we adjusted for postnatal age at blood sampling but we lacked information beyond the first days of life to examine if our results present just a transient or maybe a permanent difference in the pool of naïve lymphocytes.

6.2 Methodological considerations

Our three studies are all prospective observational cohort studies. Observational cohort studies are used to identify relationships between exposure and outcome, but cannot establish causality. Mode of delivery, i.e., elective CS compared to normal VD, was the main exposure in all three papers. Other covariates were maternal and infant characteristics, time of blood sampling *(Paper I and III)* and duration of labor *(Paper II)*. Levels of global DNA-methylation *(Paper I and II)*, locus-specific DNA-methylation (Paper II) and TREC/ KREC levels *(Paper III)* were our outcome data.

The prospective cohort study design has various advantages. The major strength is the accuracy of data collection and the possible disadvantage of a long follow-up period was not relevant for our outcome data. In context of this thesis, the most important is that the study design allows hypothesis testing, detailed and in depth exploration of molecular mechanisms, blinding to participants' exposure status, validation and replication analyses. Cohort studies can be applied if randomization is not possible which, was the case in this thesis.

Internal validity

A study has high internal validity if it is unlikely that findings are explained by systematic or random errors. A systematic error is generally referred to as bias. Bias is independent of study size but can be reduced by carefully planning and designing the study.

Selection bias

Selection bias is the bias introduced by the selection of individuals for analysis, so that the obtained sample is not representative for the participants and data intended to be analyzed.

In paper I and II, we have no population-based cohorts and participants were naturally not randomly assigned to the two modes of delivery. Parents were asked if they wanted to participate in our studies and those who gave consent and had no other exclusion criteria, were included. But we have no information about those who declined participation. Parents and infants not participating in the studies could be different from participating individuals. However, we have no obvious reason to believe that our study population in terms of delivery mode is not representative for the entire population and a possible difference would distinguish within the groups and contribute to selection bias.

The strength of study III is the population-based cohort design on a large number

of 7,174 singleton, live-born infants, delivered in a certain period of time, three month, and in a certain geographical area, Stockholm.

Information bias

Inaccurate recording and classification of exposures and outcomes can be referred to as information bias or misclassification.

In paper I and II, data were obtained from medical records, containing maternal, pregnancy, delivery, and infant data. Medical records were written by health care staff not being aware of the data collection in paper I and II.

Another strength with study I and II were that the investigators who sorted subtypes of white blood cells, extracted DNA and analyzed DNA-methylation had no information about the exposures and participant characteristics. Blinding also applied to the TREC and KREC analyses in study III.

After DNA extraction, however, not all samples contained a sufficient amount of DNA for methylation analyses, so we did not receive any outcome data (Paper II). However, we could not find associations between infants or mothers characteristics and insufficient amount of DNA. Reasons for this lower success rate were, above all, errors in blood sample management resulting in blood clotting and/or filling the test tubes with too few milliliters blood.

A limitation is that the genome-wide measurement of DNA-methylation by Illumina 450K cannot differ between methylcytosine and hydroxymethylcytosine. Even if the percentage of hydroxymethylcytosine is suggested to be lower than one percent of the methylated cytosines, we cannot exclude that it may have contributed to some of our findings. On the other hand, a strength is that we also performed validation analyses for eight CpGs in four certain genes by bisulfite pyrosequencing to confirm our findings from the 450K Illumina analysis. Using bisulfite pyrosequencing, the direction of methylation differences found in CS and VD infants was consisting with the results by Illumina *(Fig 4, paper II).* And in all three papers, the methods for measurement of DNA-methylation[132, 134, 196] and TREC/KREC[122] are validated methods.

In paper III we obtained data on mother, pregnancy, delivery and infant characteristics from the population-based Stockholm-Gotland Obstetric Database. This database contains automatically retrieved information from the medical record system used in the region for all maternity, delivery and postnatal care units. The data were forwarded daily from the medical records to the database. It included all prospectively collected and standardized information from antenatal care, all information from delivery and the postpartum period for mother and infant.[129] Data misclassification is therefore unlikely.

Confounding bias

Confounding refers to that the association between exposure and outcome is affected by a third factor related to both exposure and outcome.

In the observational studies I and II on mode of delivery and DNA-methylation, we designed our protocols to reduce confounding by using restriction. Multiple pregnancy, maternal diabetes, hypertension, preeclampsia, smoking and medication during the index pregnancy, preterm delivery and SGA infants, neonatal asphyxia, malformations, chromosomal disorders, or congenital infection were all exclusion criteria. There were some minor differences in maternal and infant characteristics between the two study groups in paper II: infants delivered by CS had on average 1 week lower GA than VD and the CS mothers were on average 3 years older. Contributions to differentially methylated CpG positions from higher maternal age and lower GA in the CS group compared to the VD group can therefore not be excluded, however, univariate analyses did not support such an interpretation.

To handle confounding related to mode of delivery and levels of TREC and KREC in the population-based cohort study III, we adjusted for risks for perinatal characteristics (mode of delivery, infant sex, gestational age, birth weight for gestational age and postnatal age at blood sampling) and for maternal characteristics (age, parity, BMI, smoking, diabetes, and hypertensive disease).

Residual confounding refers to confounding exposures that cannot be controlled for, i.e. confounding bias that remains after controlling for known/measured confounding factors in the design or analysis of a study.

Despite the well-characterized cohort and standardized study protocol in paper I and II and the large population-based cohort in study III, there are factors we could not measure or adjust for. For example, our database did not include time of cord clamping. It cannot be ruled out that timing of cord clamping - and the following postnatal placental transfusion of blood cells to the infant – may have differed in relation to mode of delivery.

The outcome in paper III was TREC and KREC levels in peripheral venous blood and not in other tissues. Accordingly, a limitation could be that the birth stress may have affected the distribution of TREC and KREC containing lymphocytes between circulation and other body compartments,[198] that we were not able to measure.

Effect modification

Effect modification or interaction is if the strength of an association changes via a third factor. The factor modifies the effect of the exposure. For example, gender is an effect-modifying factor if an exposure is associated with an outcome among

males, but not among females.

A consideration regarding effect modification in paper I was that differences in differential WBC count could have contributed to some of the observed differences in DNA-methylation between the two groups. Also, if the calculation of the ratio between methylated and unmethylated DNA takes differences in total WBC number and DNA into account. There are larger birth-related shifts in neutrophils and monocyte counts in infants born vaginally as compared to CS, whereas distributions of lymphocytes in cord blood does not relate to mode of delivery.[19, 197] In study II, we could thereafter demonstrate that isolated CD34+ hematopoietic stem cells presented significantly more methylated DNA in cells from CS infants compared to cells from VD infants. It is therefore unlikely that a different mode of birth-related shift of neutrophils had a crucial contribution to our findings in study I.

Random error

Even in the absence of bias (systematic errors), chance could explain observed findings. Random errors depend on study size. The cohort analysis in paper III is based on a large study population, minimizing the risk of chance findings. The analyses in paper I and II were based on smaller samples of study subjects, and the possibility of random errors cannot be ruled out. However, the group differences were statistically significant on a probability level < 5 % and could be replicated suggesting that probability of chance findings was minimal.

External validity

While internal validity is related to the likelihood that observations are true for the study population itself, external validity refers to if results could be generalized to other, not here studied populations. Whether research can be generalized to other contexts depend on the degree of similarity between the study population and populations in the other contexts.

Due to relatively small number of healthy infants in paper I and II, it is difficult to assess external validity. Observed findings need to be confirmed in larger studies.

Paper III was based on a large population-based cohort of singleton, live-born infants, delivered in a period of three month in a certain geographical area, implying that results are applicable to the population of newborn infants in Sweden and other high resource countries.

6.3 Ethical considerations

Study I and II were small observational cohort studies and participation required informed consent. The ethical considerations were related to the potential discomfort of blood sampling. Sampling of cord blood *(Paper I and II)* does not cause any discomfort for the infant or mother. Peripheral blood sampling of mothers *(Paper I)* was performed together with venous catheter insertion during delivery and at day 3-5 by venipuncture of an experienced research nurse. The sampling was voluntary but all mothers accepted and no extra puncture was necessary. Infants blood sampling for DNA-methylation along with sampling for neonatal screening at day 3-5 *(Paper I)* was performed by an experienced neonatal research nurse. The ethical approval allowed one extra venipuncture if parents agreed and that was only necessary in a few cases. Levels of CRP and folate *(Paper I)* were assessed by a pediatrician and no value required clinical intervention. We also made sure to always answer questions and worries from the participants.

In study III, we used register data from the Stockholm-Gotland Obstetric Database and already existing TREC- and KREC-levels, analyzed as part of a neonatal screening project to detect severe combined immune deficiencies,[122] where parents had chosen to participate. Study subjects did not to do any extra tests. Ethical considerations are related to the registration itself. Individuals could be uncomfortable knowing that there is information about them available in registers. Studies of this kind are basically only possible in the Nordic countries because of our population-based research registers. Sweden therefore has a responsibility towards the rest of the world to develop and test hypotheses that require both population-based approaches and high statistical strength. Utility should therefore considerer the potential risks of perceived integrity violation of the individuals present in the registry, who are not identified and only analyzed at group level.

All patient material in our studies has been handled with a high level of confidentiality. In all our studies, results were presented at group level and no individual result could be revealed.

The presented studies comprise any clinical outcomes and no participant could be identified as an individual at risk.

Our findings about association of delivery mode, epigenetic modifications and de novo production of neonatal lymphocytes may be perceived as controversial by the profession and by the public. Together with the other evidence presented herein, such knowledge might be able to worry and complicate women and their partner's choice and necessity of delivery mode for their children.

7 Conclusions

The following epigenetic effects were found to be related to mode of delivery:

- Global DNA-methylation in white blood cells of newborn infants delivered by elective CS was significantly higher as compared to levels seen after normal vaginal delivery.
- Levels of global DNA-methylation in CD34+ hematopoietic stem/progenitor cells of newborn infants delivered by elective CS were significantly higher as compared to those born vaginally.
- Genome-wide, locus-specific DNA methylation in CD34+ hematopoietic stem/progenitor cells differed maximal 40 percent between infants born vaginally and those by elective CS, totally in 343 CpG positions, 179 associated with known genes.
- In three specific genes, we found evidence for a relationship between duration of labor and degree of DNA-methylation.
- The GREAT analysis associated our gene set with the biological processes of immunoglobulin synthesis, metabolism and apoptosis.

Establishment of immune function at birth may be related to various perinatal risk factors, such as gestational age, mode of delivery and birth weight:

- Infants born by elective CS had a 32 percent higher risk of having low number of naïve T-lymphocytes.
- Delivery at 35 to 36 gestational weeks, being born SGA and male sex were also associated with a shift towards lower counts TREC+ T-cells.
- Infants born SGA, post-term, and of male sex were also at increased risk of low levels of B-lymphocytes (KREC values within the lowest quintile).

Given the rapidly increasing rates of elective CS worldwide and the greater risk for immune disorders later in life, our findings may have important implications for future research how mode of delivery affect the epigenetic state and birth-related surge in lymphocyte formation.

8 Future Perspectives

Associations between CS delivery and immune system related disorders later in life requires further efforts to resolve issues on underlying mechanisms, causation and counseling. Our findings may contribute with pieces of knowledge to gradually solve this puzzle. Our results have so far no direct clinical impact and current Swedish guidelines regarding indication for elective CS should not be changed. However, awareness among both physicians and pregnant women about the associations between CS and potentially poorer health in the offspring is warranted, and CS should not be recommended without an evaluation of harms and benefits, both for the mother and her infant.

Future research on CS and health effects in offspring should focus on longitudinal aspects targeting epigenetic state of specific genes with immunoregulating functions. An important task would be to investigate whether any of the DMPs associated with the mode of delivery, i.e. in the HLA-F gene, retain their methylation profile in CD34+ hematopoietic stem/progenitor cells into adulthood.

Umbilical cord blood is used for transplantation purposes. Mode of delivery affects the epigenetic state of the hematopoietic stem/progenitor cells. However, to the best of my knowledge, it is unknown if that also could affect the transplantation outcomes.

Study III identified several important risk factors for reduced number of naïve T- and B-lymphocytes in newborn infants. Future research should focus on longitudinal aspects of how, among other factors, elective CS influences the lymphocyte numbers and function in offspring. Does the lower quantity of newly matured lymphocytes relate to infections as well as disorders assigned to immune function later in childhood and adult life? We found quantity differences in naïve lymphocytes, does it affect the function as well? May there not be enough combinations of T-cell receptors, so that any subsequent invasion of microbes in the future cannot be responded to?

DNA-methylation plays undoubtedly a role in the rearrangement process of B-and T-lymphocytes and the thymic differentiation of T-lymphocytes.[125-127] Could mode of delivery via epigenetic mechanisms alter their function?

Finally, there are medical indications for CS and this should be acknowledged. Induced "stress of being born" and restoring of microbiota could pres-

ent possible interesting concepts for future research on elective CS. A small study inducing birth stress by terbutaline demonstrated better short-term adaptions of infants delivered by elective CS.[198] The microbiome of infants after elective CS exposed to maternal vaginal fluids at birth was at one month of age similar to those delivered by VD.[199] Larger studies based on these concepts regarding described long-term consequences of CS, immune system, and epigenetics are needed in this research area.

9 Svensk sammanfattning

Bakgrund

Epidemiologisk forskning har klarlagt att flera av våra stora folksjukdomar, såsom kardiovaskulära sjukdomar, metabolt syndrom och cancer, inte bara beror på arvanslag och livsstil i vuxen ålder. Även störningar i människans tidiga utveckling, som dålig fostertillväxt eller för tidig födsel, har visats spela stor roll för risken att drabbas av folksjukdomar som vuxen. Dessa relativt nya kunskaper har kommit att förändra synen på sjukdomars uppkomst.

Stressreaktionen av att födas vaginalt överskrider all annan känd fysisk stress som människan utsätts för under sin livstid. Vi vet att barnets omställning vid födseln underlättas av att fostret på detta sätt förbereds av värkarbetet. Ett barn som föds oförberett och med planerat kejsarsnitt har svårare att anpassa sig till yttervärlden lika bra och lika snabbt. Kortfristiga följder som adaptionsstörningar beträffande andning, cirkulation och hypoglykemi är välbeskrivna.

Andelen förlossningar med kejsarsnitt ökar snabbt, och i ett globalt perspektiv är kejsarsnitt numera det vanligaste kirurgiska ingreppet bland kvinnor i fertil ålder. Det är framförallt andelen planerade kejsarsnitt före värkarbetets start som tilltar, vilket gör att allt fler barn föds utan den aktivering av kroppens försvarssystem som värkarbete och vaginal förlossning medför. Samtidigt har epidemiologiska studier på senare år visat att barn och vuxna födda med kejsarsnitt löper ökad risk för sjukdomar med ofta immunologisk grund, såsom astma, allergi, typ 1 diabetes, inflammatorisk tarmsjukdom, vissa typer av cancer, men också fetma och autism. Orsakerna till dessa samband är oklara.

Epigenetik är läran om hur samma genetiska information i alla kroppens celler, kodad i DNA, uttrycks olika beroende på cellens funktion. Epigenetiska mekanismer reglerar aktivering eller repression av gener utan att förändra DNA sekvensen. DNA-metylering är den mest basala epigenetiska modifieringen. Metylering sker nästan uteslutande på cytosin som följs av guanin (CpG). Andra epigenetiska mekanismer är modifieringar av histoner, såsom fosforylering, acetylering mm och förekomst av icke-kodande RNA (RNA som inte direkt leder till ett protein). Epigenetiska förändringar kan orsaka eller bero på sjukdom.

Djurförsök och studier hos människan pekar på att stressen vid födelsen och under nyföddhetsperioden kan förändra den epigenetiska koden och kan leda till

ändrade hormonnivåer av tex kortisol och minskad stresstålighet.

T-och B-lymfocyter är del av det adaptiva immunsystemet och utvecklas från hematopoetiska stamceller via progenitor celler i benmärgen. T-celler utvandrar till thymus och mognar där, medan B-celler förblir i benmärgen. Båda utvecklar antigenspecifitet genom en process kallad för slumpmässig omarrangering (rearrangement). Under denna process bildas bi-produkter kallade TREC och KREC. Genom mätning av TREC och KREC kan man få en uppfattning av hur många mogna T-och B-lymfocyter som förekommer i blodet. Ungefär tre fjärdedelar av alla naiva lymfocyter produceras i samband med och upp till en vecka efter förlossningen.

Syfte

Det övergripande syftet med denna avhandling var att studera om förlossningssätt och andra perinatala faktorer påverkar det nyfödda barnets epigenetiska och immunologiska tillståndet. De specifika målsättningarna var:

- att studera graden av global DNA-metylering i vita blodkroppar i blodprover från navelsträng och perifer ven (dag 3 till 5) hos friska nyfödda barn förlösta vaginalt eller med planerat kejsarsnitt
- att studera graden av global och lokusspecifik DNA-metylering i hematopoetiska (CD34+) stam- och progenitor celler från navelsträngsblod hos friska nyfödda barn förlösta vaginalt eller med planerat kejsarsnitt
- att studera antalet mogna, naiva T- och B-lymfocyter hos friska nyfödda barn i förhållande till förlossningssätt, graviditetslängd och födelsevikt.

Material och metoder

Delarbete I and II är prospektiva observationsstudier av 37 (planerat kejsarsnitt =16) respektive 64 (planerat kejsarsnitt =27) friska nyfödda barn. Blodprover togs från navelsträngen och i studie I, även perifert på dag 3 till 5 efter födseln i samband med nyföddhetsscreeningen. Global DNA-metylering mättes i vita blodkroppar med hjälp av metoden ”luminometric methylation assay” (LUMA). I studie II mättes global and genomvid, lokusspecifik DNA-metylering i hematopoetiska (CD 34+) stam- och progenitor celler med hjälp av metoderna LUMA och Illumina Infinium 450K. Validering skedde genom bisulfit-pyrosekvensering.

I delarbete III använde vi oss av prospektivt insamlade data från databasen Stockholm-Gotland Obstetric Data Base, innehållande barnets, moderns och förlossningens karaktäristika från 6.014 friska, nyförlösta barn in Stockholm. Dessa data länkades ihop med värden för T-cell receptor excision circles (TREC) och κ-deleting recombination excision circles (KREC), som mättes som del av

nyföddhetsscreeningen för immunbristsjukdomar. TREC och KREC värden representerar mängden av mogna, naiva T- och B-lymfocyter.

I samtliga delarbeten gjordes statistiska beräkningar i syfte att kunna säkerställa icke-slumpmässiga skillnader i metyleringsgrad och lymfocyttal i förhållande till exponeringsdata.

Delarbete I

Global DNA-metylering i vita blodkroppar från navelsträngen var ökat om man förlöstes med planerat kejsarsnitt jämfört med vanlig vaginal förlossning ($p < 0.001$). DNA-metylering i leukocyter tagna i samband med nyföddhetsscreeningen dag 3 till 5 efter förlossningen visade inte längre någon statistisk skillnad mellan dessa två grupper. Det fanns ingen korrelation mellan DNA-metylering och moderns, barnets samt övriga perinatala riskfaktorer.

Delarbete II

Global DNA-metylering i hematopoetiska stam- och progenitor celler från navelsträngen var ökat om man förlöstes med planerat kejsarsnitt jämfört med vanlig vaginal förlossning ($p = 0.02$). Det fanns ingen korrelation mellan global DNA-metylering och moderns, barnets samt övriga perinatala riskfaktorer.

Lokusspecifik DNA-metylering i CD34+celler visade en skillnad på > 10 % i 342 CpG dinukleotider ($p < 0.01$). Största skillnaden var ca 40 % och 179 av 342 olikmetylerade ställen var associerade med kända gener. DNA-metyleringen i tre CpG nukleotider visade ett samband med längden av värkarbetet. Valideringsanalysen med bisulfitpyrosekvensering av metyleringsskillnader i CpG dinukleotider associerat med fyra kända gener, bekräftade fynden från Illumina Infinium 450K-analysen. De genregioner med olikmetylerade CpG dinukleotider som vi fann, ingår i biologiska processer som omfattar immunglobulin syntesen, metabolism och apoptos.

Delarbete III

Upprättandet av immunfunktion vid födseln kan relateras till olika perinatala riskfaktorer såsom förlossningssätt, gestationsålder, kön och födelsevikt. Friska nyfödda barn förlösta med planerat CS hade en 32 procent högre risk för att ha lägre antal nybildade, naiva T-lymfocyter. Även barn födda i gestationsvecka 35 och 36, födda för lätta för gestationsåldern och av manlig kön uppvisade risken för lägre antal TREC-positiva lymfocyter. Dessutom hade nyfödda barn för små för gestationsålder och av manligt kön en förhöjd risk för lägre antal KREC-positiva lymfocyter.

Inga av fynden i delarbete I-III visar något orsakssamband mellan förlossningssätt och sjukdomar senare i livet och den kliniska innebörden behöver undersökas i fortsatta studier.

Slutsatser

Sammanfattningsvis demonstrerar fynden från våra arbeten att förlossningssätt påverkar epigenetiska modifieringar i form av DNA-metylering globalt i vita blodkroppar och hematopoetiska stam- och progenitorceller samt även genomvid, lokusspecifik i dessa CD34+ celler. Vårt senaste arbete ger för första gången också belägg för att förlossningssätt, kön, gestationsålder och födelsevikt i förhållande till gestationsålder påverkar upprättandet av det adaptiva immunförsvaret i form av ett lägre antal mogna, naiva T-och/eller B-lymfocyter.

10 Acknowledgements

It would not have been possible to complete this thesis without encouragement, assistance and contributions from many people. First, I would like to express my gratitude to all parents and their newborn children for participating in study I and II in this thesis. I also would like to acknowledge infants and their mothers appearing as anonymous "observations" in the datasets used in paper III.

In particular, I would like to thank:

Mikael Norman, main supervisor and friend. I can't imagine a better mentor than you, from research to clinical work. You are an exceptional researcher, always with a solution at hand. I have learned so much from you. Thank you for always supporting me, both in professional and personal life. You are always a telephone call away.

Stefan Johansson, co-supervisor, friend and neighbor. Not just for all your wise guidance, statistical work and support, especially you are a great epidemiologist and researcher. An inspiration to us all with your exciting ideas and varying ongoing projects. More importantly, you are a great friend and I appreciate all the time we spend together on our favorite islands.

Tomas Ekström, co-supervisor and excellent epigenetic researcher, for helping my epigenetic ball hopefully fall into the right valley. Thank you for your patience in the time-consuming task of guiding and teaching me when my knowledge of DNA-methylation and epigenetics has fallen short. Thanks to you, I have always felt that there is no such thing as a stupid question.

Kerstin Wicknertz, my external mentor, friend, and clinical supervisor during the first two years of residency in pediatrics at Mälarsjukhuset Eskilstuna. I have really appreciated our talks throughout the years. You are a great role model.

The co-authors in our epigenetic studies: A***gneta Gunnar***, always ready to receive samples and able to run DNA extraction and LUMA at all possible and impossible times. ***Malin Almgren***, for great collaboration, and above all, for making Illumina understandable to me. ***Mikael Sundin***, not only for all the work in paper II, but more importantly your friendliness and never-ending patience answering my questions about immunology, stem cells, lymphocytes and brainstorming new ideas. ***David Gomez-Cabrero***, for fantastic biostatistical work.

Lennart Hammarström, ***Olof Stephansson***, ***Rolf Zetterström***, S***ven Cnattingius***, and ***Ulrika von Döbeln***, co-authors in our study about immune cell formation. I have appreciated our collaboration.

Jessica Schiött, for taking the blood samples at newborn screening in an elegant way.

All ***midwifes***, ***nurses,*** and ***assistant nurses*** at BB Stockholm and department 17 at Danderyds hospital and maternity ward Karolinska Huddinge, for the sampling of cord blood. Without you, it would not have been possible!

Henric Lindqvist, for cell separation and ***Fatmire Bujupi,*** for contribution in the validation process in study II. ***Gunnar Petersson***, for managing the databases and ***Michela Barbaro***, for organizing the screening data in study III.

Mats Blennow, head of CLINTEC and former clinical mentor during my residency in neonatology, not only for your great scientific support but also all clinical encouragement.

Claude Marcus, head of the Division of Pediatrics at CLINTEC, for creating a scientific environment. I also would like to thank the administrative staff at CLINTEC, especially ***Agneta Wittlock, Lisbeth Sjödin***, and ***Maria Staiger***, for invaluable help with practical matters.

My bosses and former bosses, ***Katarina von Schewelow***, ***Peter Larsson***, J***onas Berner***, ***Mikael Rolfs***, ***Inger Mossberg***, ***Anders Wallin***, ***Nina Perrin***, ***Ann-Britt Bohlin***, ***Baldvin Jonsson*** and ***Eva Berggren Broström***. Thank you for your encouragement and for creating a research-friendly environment!

Lena Påhlsson and ***Lena Centerwall*** at barn PMI, for invaluable help with practical matters.

My colleagues at the department for children´s anesthesia and intensive care. ***Urban***, thank you for recruiting me. From the start, I enjoyed everyone's support, encouragement and amicability. Just to mention some of you: ***Tova***, judicious clinical supervisor during my residency in anesthesia, ***Jarl***, my unofficial mentor in anesthesia. ***Magnus***, for making it possible to catch my flights, my former roommates, ***Andreas, Björn, Ivan, Johannes***, and ***Ulf. Märit,*** for proofreading and wise comments. ***Martin***, making writing time for me in the schedule. Last but not least for the positive attitude of all of you!

All former and current colleagues at the Departments of pediatrics, neonatology, anesthesia, and thoracic anesthesia in Eskilstuna, Uppsala, at Karolinska, and Narkoskliniken Stockholm. I really enjoy working with you!

Everyone who deserves being mentioned by name.

PA Lönnqvist, for welcoming me to your research group. ***Marie Olofsson***, colleague and friend, from neonatology to anesthesiology and doctoral thesis.

Sten Swanström, for introducing me to neonatal clinical work and research. ***Uwe Ewald***, for always asking the tricky questions. ***Hugo Lagercrantz***, for all support.

The NeoProg research group members, for sharing your research, ideas and coming with valuable input.

Ulf Johansson, many thanks for the esthetic layout and patience with my changes.

Tomas Heitzer, main supervisor of my dissertation at UKE in Hamburg. For all your encouragement and introducing me to clinical research.

Mathias, comrade and colleague, for all the long walks and stops at Djurgården during paternity leave.

Meinen Geschwistern mit Familien, ***Fabiola*** und ***Livius***, für Ihr seid die besten.

Meinen Eltern, ***Ulrike*** und ***Eckhart,*** für uns die Freiheit im Leben gegeben zu haben, machen zu können was wir wollen, nur wir werden glücklich damit.

My own modern family with all fantastic ”bonusbarn” ***Sarah, Julia, Elsa***, and ***Oskar*** (Oskar, thank you so much for proofreading and all wise comments!). ***Kalle*** and ***Gisela***, for nice and supportive company at any time.

And finally, my wife ***Len***a, and our wonderful son, ***August***. Lena, I cannot thank you enough. Your great effort as a research nurse must be mentioned, which is just a small part of everything I should thank you for. Life is good, forward to new adventures! I love you!

The papers included in this thesis were generously supported by Karolinska Institutet´s research foundations, by a regional agreement on clinical research between Stockholm County Council and Karolinska Institutet, by grants from the Swedish Order of Freemason´s Foundation for Children´s Welfare, the American Liver Foundation, the Samariten Foundation in Stockholm, the Swedish Society for Medical Research (SSMF), the Swedish Research Council, and the Strategic Research Program in Epidemiology at Karolinska Institutet.

11 References

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1(8489):1077-81. Epub 1986/05/10. PubMed PMID: 2871345.
2. Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, et al. Glucose tolerance in adults after prenatal exposure to famine. Lancet. 1998;351(9097):173-7. Epub 1998/02/05. PubMed PMID: 9449872.
3. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science. 2004;305(5691):1733-6. Epub 2004/09/18. doi: 10.1126/science.1095292. PubMed PMID: 15375258.
4. Hanson M, Gluckman P. Early developmental conditioning of later health and disease: physiology or pathophysiology? Physiological reviews. 2014;94(4):1027-76.
5. Gluckman PD, Hanson MA, Low FM. The role of developmental plasticity and epigenetics in human health. Birth Defects Research Part C: Embryo Today: Reviews. 2011;93(1):12-8.
6. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol. 2003;23(15):5293-300. Epub 2003/07/16. PubMed PMID: 12861015; PubMed Central PMCID: PMCPMC165709.
7. Morgan HD, Sutherland HG, Martin DI, Whitelaw E. Epigenetic inheritance at the agouti locus in the mouse. Nature genetics. 1999;23(3):314.
8. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nature neuroscience. 2004;7(8):847-54. PubMed PMID: 15220929.
9. McGowan PO, Sasaki A, Huang TC, Unterberger A, Suderman M, Ernst C, et al. Promoter-wide hypermethylation of the ribosomal RNA gene promoter in the suicide brain. PloS one. 2008;3(5):e2085. doi: 10.1371/journal.pone.0002085. PubMed PMID: 18461137; PubMed Central PMCID: PMC2330072.
10. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics. 2008;3(2):97-106. PubMed PMID: 18536531.

11. Demetriou CA, Veldhoven K, Relton C, Stringhini S, Kyriacou K, Vineis P. Biological embedding of early-life exposures and disease risk in humans: a role for DNA methylation.
European journal of clinical investigation. 2015;45(3):303-32.
12. Lagercrantz H. The good stress of being born.
Acta paediatrica. 2016;105(12):1413-6. doi: 10.1111/apa.13615.
13. Lagercrantz H, Slotkin TA. The "stress" of being born. S
cientific American. 1986;254(4):100-7. PubMed PMID: 3961465.
14. Irestedt L, Lagercrantz H, Hjemdahl P, Hagnevik K, Belfrage P. Fetal and maternal plasma catecholamine levels at elective cesarean section under general or epidural anesthesia versus vaginal delivery.
American journal of obstetrics and gynecology. 1982;142(8):1004-10.
Epub 1982/04/15. PubMed PMID: 7072768.
15. Finley N, Norlin A, Baines DL, Folkesson HG. Alveolar epithelial fluid clearance is mediated by endogenous catecholamines at birth in guinea pigs.
The Journal of clinical investigation. 1998;101(5):972-81.
16. Hagnevik K, Faxelius G, Irestedt L, Lagercrantz H, Lundell B, Persson B. Catecholamine surge and metabolic adaptation in the newborn after vaginal delivery and caesarean section.
Acta Paediatr Scand. 1984;73(5):602-9. Epub 1984/09/01.
PubMed PMID: 6485778.
17. Gitau R, Menson E, Pickles V, Fisk NM, Glover V, MacLachlan N. Umbilical cortisol levels as an indicator of the fetal stress response to assisted vaginal delivery.
European Journal of Obstetrics & Gynecology and Reproductive Biology.
2001;98(1):14-7.
18. Christensson K, Siles C, Cabrera T, Belaustequi A, de la Fuente P, Lagercrantz H, et al. Lower body temperatures in infants delivered by caesarean section than in vaginally delivered infants.
Acta paediatrica. 1993;82(2):128-31. Epub 1993/02/01.
PubMed PMID: 8477157.
19. Yektaei-Karin E, Moshfegh A, Lundahl J, Berggren V, Hansson LO, Marchini G. The stress of birth enhances in vitro spontaneous and IL-8-induced neutrophil chemotaxis in the human newborn. Pediatric Allergy and Immunology. 2007;18(8):643-51.
20. Lagercrantz H, Pequignot J, Pequignot JM, Peyrin L. The first breaths of air stimulate noradrenaline turnover in the brain of the newborn rat.
Acta Physiol Scand. 1992;144(4):433-8.
Epub 1992/04/01. doi: 10.1111/j.1748-1716.1992.tb09317.x.
PubMed PMID: 1605045.
21. Statistics on Pregnancies, Deliveries and Newborn Infants 2015, www.socialstyrelsen.se/publikationer2017/2017-3-3, 2017-03-21, ISSN 1400-3511.

22. Bulger T, Howden-Chapman P, Stone P. A cut above: the rising Caesarean section rate in New Zealand.
The New Zealand medical journal. 1998;111(1059):30-3.
23. Menacker F, Declercq E, Macdorman MF, editors. Cesarean delivery: background, trends, and epidemiology. Seminars in perinatology; 2006: Elsevier.
24. Boatin AA, Schlotheuber A, Betran AP, Moller A-B, Barros AJ, Boerma T, et al. Within country inequalities in caesarean section rates: observational study of 72 low and middle income countries. BMJ. 2018;360:k55.
25. Low J. Caesarean section--past and present. J Obstet Gynaecol Can. 2009;31(12):1131-6. Epub 2010/01/21. PubMed PMID: 20085678.
26. Hogberg U. Maternal deaths related to cesarean section in Sweden, 1951-1980. Acta Obstet Gynecol Scand. 1989;68(4):351-7. Epub 1989/01/01. PubMed PMID: 2618623.
27. Ye J, Zhang J, Mikolajczyk R, Torloni M, Gülmezoglu A, Betran A. Association between rates of caesarean section and maternal and neonatal mortality in the 21st century: a worldwide population-based ecological study with longitudinal data.
BJOG: An International Journal of Obstetrics & Gynaecology. 2015.
28. Molina G, Weiser TG, Lipsitz SR, Esquivel MM, Uribe-Leitz T, Azad T, et al. Relationship between cesarean delivery rate and maternal and neonatal mortality. Jama. 2015;314(21):2263-70.
29. Althabe F, Sosa C, Belizán JM, Gibbons L, Jacquerioz F, Bergel E. Cesarean section rates and maternal and neonatal mortality in low-, medium-, and high-income countries: an ecological study. Birth. 2006;33(4):270-7.
30. Mascarello KC, Matijasevich A, Barros AJ, Santos IS, Zandonade E, Silveira MF. Repeat cesarean section in subsequent gestation of women from a birth cohort in Brazil. Reproductive Health. 2017;14(1):102.
31. Franz MB, Husslein PW. Obstetrical management of the older gravida. Womens Health (Lond). 2010;6(3):463-8.
Epub 2010/04/30. doi: 10.2217/whe.10.26. PubMed PMID: 20426610.
32. Mylonas I, Friese K. Indications for and risks of elective cesarean section. Deutsches Ärzteblatt International. 2015;112(29-30):489.
33. Gillet E, Martens E, Martens G, Cammu H. Prelabour caesarean section following IVF/ICSI in older-term nulliparous women: too precious to push? J Pregnancy. 2011;2011:362518. Epub 2011/12/02. doi: 10.1155/2011/362518. PubMed PMID: 22132336; PubMed Central PMCID: PMCPMC3216354.
34. Nieminen K, Stephansson O, Ryding EL. Women's fear of childbirth and preference for cesarean section – a cross-sectional study at various stages of pregnancy in Sweden. Acta Obstetricia et Gynecologica Scandinavica. 2009;88(7):807-13. doi: 10.1080/00016340902998436.

35. Lee YM, D'Alton ME. Cesarean delivery on maternal request: maternal and neonatal complications. Current opinion in obstetrics & gynecology. 2008;20(6):597-601. PubMed PMID: 18989137.

36. Tita AT, Landon MB, Spong CY, Lai Y, Leveno KJ, Varner MW, et al. Timing of elective repeat cesarean delivery at term and neonatal outcomes. New England Journal of Medicine. 2009;360(2):111-20.

37. De Luca R, Boulvain M, Irion O, Berner M, Pfister RE. Incidence of early neonatal mortality and morbidity after late-preterm and term cesarean delivery. Pediatrics. 2009;123(6):e1064-71.
Epub 2009/06/02. doi: 10.1542/peds.2008-2407. PubMed PMID: 19482739.

38. Liston FA, Allen VM, O'Connell CM, Jangaard KA. Neonatal outcomes with caesarean delivery at term. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2008;93(3):F176-F82.

39. Jain L, Dudell GG, editors. Respiratory transition in infants delivered by cesarean section.
Seminars in perinatology; 2006: Elsevier.

40. Cho CE, Norman M. Cesarean section and development of the immune system in the offspring.
American journal of obstetrics and gynecology. 2013;208(4):249-54.

41. Gialloreti LE, Benvenuto A, Benassi F, Curatolo P. Are caesarean sections, induced labor and oxytocin regulation linked to Autism Spectrum Disorders? Med Hypotheses. 2014;82(6):713-8.
Epub 2014/04/02. doi: 10.1016/j.mehy.2014.03.011. PubMed PMID: 24685110.

42. Kuhle S, Tong OS, Woolcott CG. Association between caesarean section and childhood obesity: a systematic review and meta-analysis.
Obesity Reviews. 2015;16(4):295-303. doi: 10.1111/obr.12267.

43. 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.

44. Polo-Kantola P, Lampi KM, Hinkka-Yli-Salomäki S, Gissler M, Brown AS, Sourander A. Obstetric Risk Factors and Autism Spectrum Disorders in Finland.
The Journal of pediatrics. 2014;164(2):358-65. doi: https://doi.org/10.1016/j.jpeds.2013.09.044.

45. Thavagnanam S, Fleming J, Bromley A, Shields MD, Cardwell CR. A meta-analysis of the association between Caesarean section and childhood asthma.
Clin Exp Allergy. 2008;38(4):629-33. doi: 10.1111/j.1365-2222.2007.02780.x. PubMed PMID: 18352976.

46. Huang L, Chen Q, Zhao Y, Wang W, Fang F, Bao Y. Is elective cesarean section associated with a higher risk of asthma? A meta-analysis.
Journal of Asthma. 2015;52(1):16-25. doi: 10.3109/02770903.2014.952435.

47. Sevelsted A, Stokholm J, Bønnelykke K, Bisgaard H. Cesarean section and chronic immune disorders. Pediatrics. 2015;135(1):e92-e8.

48. Renz-Polster H, David MR, Buist AS, Vollmer WM, O'Connor EA, Frazier EA, et al. Caesarean section delivery and the risk of allergic disorders in childhood.
Clinical & Experimental Allergy. 2005;35(11):1466-72. doi: 10.1111/j.1365-2222.2005.02356.x.

49. Pistiner M, Gold DR, Abdulkerim H, Hoffman E, Celedon JC. Birth by cesarean section, allergic rhinitis, and allergic sensitization among children with a parental history of atopy. T
he Journal of allergy and clinical immunology. 2008;122(2):274-9.
PubMed PMID: 18571710.

50. Koplin J, Allen K, Gurrin L, Osborne N, Tang ML, Dharmage S. Is caesarean delivery associated with sensitization to food allergens and IgE-mediated food allergy: a systematic review.
Pediatr Allergy Immunol. 2008;19(8):682-7. PubMed PMID: 19076564.
Epub 2008/12/17. doi: 10.1111/j.1399-3038.2008.00731.x.

51. Sánchez-Valverde F, Gil F, Martinez D, Fernandez B, Aznal E, Oscoz M, et al. The impact of caesarean delivery and type of feeding on cow's milk allergy in infants and subsequent development of allergic march in childhood. Allergy. 2009;64(6):884-9. doi: 10.1111/j.1398-9995.2008.01931.x.

52. Cardwell CR, Stene LC, Joner G, Cinek O, Svensson J, Goldacre MJ, et al. Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies. Diabetologia. 2008;51(5):726-35. PubMed PMID: 18292986.

53. Håkansson S, Källén K. Caesarean section increases the risk of hospital care in childhood for asthma and gastroenteritis.
Clinical & Experimental Allergy. 2003;33(6):757-64.

54. Decker E, Engelmann G, Findeisen A, Gerner P, Laaβ M, Ney D, et al. Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. Pediatrics. 2010;125(6):e1433-e40.

55. McLaughlin CC, Baptiste MS, Schymura MJ, Zdeb MS, Nasca PC. Perinatal risk factors for neuroblastoma.
Cancer Causes & Control. 2009;20(3):289-301.

56. Cook MB, Graubard BI, Rubertone MV, Erickson RL, McGlynn KA. Perinatal factors and the risk of testicular germ cell tumors. International journal of cancer. 2008;122(11):2600-6. PubMed PMID: 18324625.

57. Thomopoulos TP, Skalkidou A, Dessypris N, Chrousos G, Karalexi MA, Karavasilis TG, et al. Prelabor cesarean delivery and early-onset acute childhood leukemia risk. European Journal of Cancer Prevention. 2016;25(2):155-61. doi: 10.1097/cej.0000000000000151. PubMed PMID: 00008469-201603000-00010.

58. Cnattingius S, Zack M, Ekbom A, Gunnarskog J, Linet M, Adami HO. Prenatal and neonatal risk factors for childhood myeloid leukemia. Cancer Epidemiol Biomarkers Prev. 1995;4(5):441-5. PubMed PMID: 7549797.

59. Momen NC, Olsen J, Gissler M, Cnattingius S, Li J. Delivery by caesarean section and childhood cancer: a nationwide follow-up study in three countries. BJOG: An International Journal of Obstetrics & Gynaecology. 2014;121(11):1343-50. doi: 10.1111/1471-0528.12667.

60. Cnattingius S, Zack MM, Ekbom A, Gunnarskog J, Kreuger A, Linet M, et al. Prenatal and neonatal risk factors for childhood lymphatic leukemia. Journal of the National Cancer Institute. 1995;87(12):908-14.

61. Bahmanyar S, Montgomery SM, Weiss RJ, Ekbom A. Maternal smoking during pregnancy, other prenatal and perinatal factors, and the risk of Legg-Calve-Perthes disease. Pediatrics. 2008;122(2):e459-e64.

62. Li Ht, Zhou Yb, Liu Jm. The impact of cesarean section on offspring overweight and obesity: a systematic review and meta-analysis. International Journal Of Obesity. 2012;37:893. doi: 10.1038/ijo.2012.195.

63. Lavin T, Preen DB. Investigating Caesarean Section Birth as a Risk Factor for Childhood Overweight. Childhood obesity (Print). 2018;14(2):131-8. Epub 2018/02/08. doi: 10.1089/chi.2017.0034. PubMed PMID: 29412743.

64. Huh SY, Rifas-Shiman SL, Zera CA, Edwards JWR, Oken E, Weiss ST, et al. Delivery by caesarean section and risk of obesity in preschool age children: a prospective cohort study. Archives of disease in childhood. 2012;97(7):610-6. doi: 10.1136/archdischild-2011-301141.

65. Mueller NT, Whyatt R, Hoepner L, Oberfield S, Dominguez-Bello MG, Widen EM, et al. Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. International journal of obesity (2005). 2015;39(4):665-70. doi: 10.1038/ijo.2014.180. PubMed PMID: PMC4390478.

66. Strachan DP. Hay fever, hygiene, and household size. Bmj. 1989;299(6710):1259-60. Epub 1989/11/18. PubMed PMID: 2513902; PubMed Central PMCID: PMCPMC1838109.

67. Neu J, Rushing J. Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis. Clin Perinatol. 2011;38(2):321-31. Epub 2011/06/08. doi: 10.1016/j.clp.2011.03.008. PubMed PMID: 21645799; PubMed Central PMCID: PMCPMC3110651.

68. 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 newborns. Proceedings of the National Academy of Sciences. 2010;107(26):11971-5.

69. Prior E, Santhakumaran S, Gale C, Philipps LH, Modi N, Hyde MJ. Breastfeeding after cesarean delivery: a systematic review and meta-analysis of world literature.
The American journal of clinical nutrition. 2012:ajcn. 030254.

70. Ly NP, Ruiz-Pérez B, Onderdonk AB, Tzianabos AO, Litonjua AA, Liang C, et al. Mode of delivery and cord blood cytokines: a birth cohort study. Clinical and Molecular Allergy. 2006;4(1):13.

71. Waddington CH. Towards a Theoretical Biology. Edinburgh, Scotland: Edinburgh University Press; 1968. The Basic Ideas of Biology; pp. 1–32.

72. Dennis C. Epigenetics and disease: Altered states.
Nature. 2003;421(6924):686-8. PubMed PMID: 12610592.

73. Reik W, Dean W. Back to the beginning. Nature. 2002;420(6912):127. PubMed PMID: 12432368.

74. Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000;403(6765):41-5. PubMed PMID: 10638745.

75. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome.
Science. 2015;347(6220):1260419.

76. Wu C, Morris JR. Genes, genetics, and epigenetics: a correspondence. Science. 2001;293(5532):1103-5. PubMed PMID: 11498582.
Epub 2001/08/11. doi: 10.1126/science.293.5532.1103.

77. Dupont C, Armant DR, Brenner CA. Epigenetics: Definition, Mechanisms and Clinical Perspective.
Seminars in reproductive medicine. 2009;27(5):351-7. doi: 10.1055/s-0029-1237423. PubMed PMID: PMC2791696.

78. Kaikkonen MU, Lam MT, Glass CK. Non-coding RNAs as regulators of gene expression and epigenetics.
Cardiovascular research. 2011;90(3):430-40.

79. Schubeler D. Function and information content of DNA methylation. Nature. 2015;517(7534):321-7.

80. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247-57. Epub 1999/11/11. PubMed PMID: 10555141.

81. Smith ZD, Meissner A. DNA methylation: roles in mammalian development. Nature Reviews Genetics. 2013;14(3):204-20.

82. Métivier R, Gallais R, Tiffoche C, Le Péron C, Jurkowska RZ, Carmouche RP, et al. Cyclical DNA methylation of a transcriptionally active promoter. Nature. 2008;452(7183):45-50.

83. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. Journal of molecular biology. 1987;196(2):261-82.

84. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes & development. 2011;25(10):1010-22.

85. Bird A. DNA methylation patterns and epigenetic memory.
Genes & development. 2002;16(1):6-21.

86. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters.
Proceedings of the National Academy of Sciences. 2006;103(5):1412-7. doi: 10.1073/pnas.0510310103.

87. McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, et al. 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events.
Mol Endocrinol. 2000;14(4):506-17. Epub 2000/04/19. doi: 10.1210/mend.14.4.0438. PubMed PMID: 10770488.

88. Razin A. CpG methylation, chromatin structure and gene silencing-a three-way connection.
Embo j. 1998;17(17):4905-8. Epub 1998/09/02. doi: 10.1093/emboj/17.17.4905. PubMed PMID: 9724627; PubMed Central PMCID: PMCPMC1170819.

89. Blackwood EM, Kadonaga JT. Going the distance: a current view of enhancer action. Science. 1998;281(5373):60-3. Epub 1998/07/25.
PubMed PMID: 9679020.

90. Bulger M, Groudine M. Functional and Mechanistic Diversity of Distal Transcription Enhancers.
Cell. 2011;144(3):327-39. doi: https://doi.org/10.1016/j.cell.2011.01.024.

91. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene.
Nature. 2000;405:482. doi: 10.1038/35013100.

92. Hahn MA, Szabó PE, Pfeifer GP. 5-Hydroxymethylcytosine: A stable or transient DNA modification?
Genomics. 2014;104(5):314-23. doi: https://doi.org/10.1016/j.ygeno.2014.08.015.

93. Jin S-G, Wu X, Li AX, Pfeifer GP. Genomic mapping of 5-hydroxymethylcytosine in the human brain.
Nucleic Acids Research. 2011;39(12):5015-24. doi: 10.1093/nar/gkr120.

94. Verdone L, Agricola E, Caserta M, Di Mauro E. Histone acetylation in gene regulation. Briefings in Functional Genomics. 2006;5(3):209-21.

95. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays. 1998;20(8):615-26.

96. Sawicka A, Seiser C. Sensing core histone phosphorylation — A matter of perfect timing().
Biochimica et Biophysica Acta. 2014;1839(8):711-8. doi: 10.1016/j.bbagrm.2014.04.013. PubMed PMID: PMC4103482.

97. Goldstein G, Scheid M, Hammerling U, Schlesinger D, Niall H, Boyse E. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proceedings of the National Academy of Sciences. 1975;72(1):11-5.

98. Cao J, Yan Q. Histone Ubiquitination and Deubiquitination in Transcription, DNA Damage Response, and Cancer. Frontiers in Oncology. 2012;2:26. doi: 10.3389/fonc.2012.00026. PubMed PMID: PMC3355875.

99. John M. Oropello, Vlad Kvetan, Stephen M. Pastores: Lange Critical Care 1st Edition, 2016, chapter 88, ISBN-13: 978-0071820813.

100. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nature reviews Molecular cell biology. 2005;6(5):376-85.

101. Kim DH, Sætrom P, Snøve O, Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. Proceedings of the National Academy of Sciences. 2008;105(42):16230-5.

102. Parkin J, Cohen B. An overview of the immune system. The Lancet. 2001;357(9270):1777-89.

103. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Laboratory investigation. 2000;80(5):617.

104. Basha S, Surendran N, Pichichero M. Immune Responses in Neonates. Expert review of clinical immunology. 2014;10(9):1171-84. doi: 10.1586/1744666X.2014.942288. PubMed PMID: PMC4407563.

105. Palmer AC. Nutritionally mediated programming of the developing immune system. Adv Nutr. 2011;2(5):377-95. Epub 2012/02/15. doi: 10.3945/an.111.000570. PubMed PMID: 22332080; PubMed Central PMCID: PMCPMC3183589.

106. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol. 2007;7(5):379-90. Epub 2007/04/26. doi: 10.1038/nri2075. PubMed PMID: 17457344.

107. Guilmot A, Hermann E, Braud VM, Carlier Y, Truyens C. Natural killer cell responses to infections in early life. J Innate Immun. 2011;3(3):280-8. Epub 2011/03/18. doi: 10.1159/000323934. PubMed PMID: 21411972.

108. Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. The Journal of immunology. 2007;179(3):1969-78.

109. Mikkola HKA, Orkin SH. The journey of developing hematopoietic stem cells. Development. 2006;133(19):3733-44. doi: 10.1242/dev.02568.

110. Forsberg EC, Bhattacharya D, Weissman IL. Hematopoietic stem cells. Stem Cell Reviews. 2006;2(1):23-30. doi: 10.1007/s12015-006-0005-z.

111. Attema JL, Papathanasiou P, Forsberg EC, Xu J, Smale ST, Weissman IL. Epigenetic characterization of hematopoietic stem cell differentiation using miniChIP and bisulfite sequencing analysis. Proceedings of the National Academy of Sciences. 2007;104(30):12371-6. doi: 10.1073/pnas.0704468104.

112. Maier H, Colbert J, Fitzsimmons D, Clark DR, Hagman J. Activation of the early B-cell-specific mb-1 (Ig-α) gene by Pax-5 is dependent on an unmethylated Ets binding site. Molecular and Cellular Biology. 2003;23(6):1946-60.

113. Maier H, Ostraat R, Gao H, Fields S, Shinton SA, Medina KL, et al. Early B cell factor cooperates with Runx1 and mediates epigenetic changes associated with mb-1 transcription. Nature immunology. 2004;5(10):1069.

114. Allis CD, Jenuwein T, Reinberg D. Epigenetic Control of Lymphopoiesis, Epigenetics: CSHL Press; 2007 page 397.

115. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human αβ T cell receptor diversity. Science. 1999;286(5441):958-61.

116. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. Journal of molecular medicine. 2001;79(11):631-40.

117. Verschuren MC, Wolvers-Tettero IL, Breit TM, Noordzij J, van Wering ER, van Dongen JJ. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. Journal of immunology (Baltimore, Md : 1950). 1997;158(3):1208-16. Epub 1997/02/01. PubMed PMID: 9013961.

118. Cahill RN, Kimpton WG, Washington EA, Dudler L, Trnka Z. An immune system switch in T cell lifespan at birth results in extensive loss of naive fetal T cells during the first week of postnatal life. Int Immunol. 1997;9(9):1253-8. Epub 1997/10/06. PubMed PMID: 9310828.

119. Hazenberg MD, Borghans JA, de Boer RJ, Miedema F. Thymic output: a bad TREC record. Nature immunology. 2003;4(2):97-9.

120. Lang PO, Govind S, Aspinall R. Reversing T cell immunosenescence: Why, who, and how 2012.

121. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med. 2007;204(3):645-55. Epub 2007/02/22. doi: 10.1084/jem.20060964. PubMed PMID: 17312005; PubMed Central PMCID: PMCPMC2137914.

122. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, et al. Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden—a 2-Year Pilot TREC and KREC Screening Study. Journal of Clinical Immunology. 2017;37(1):51-60.

123. King JR, Hammarström L. Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. Journal of Clinical Immunology. 2018;38(1):56-66. doi: 10.1007/s10875-017-0455-x.

124. van der Spek J, Groenwold RHH, van der Burg M, van Montfrans JM. TREC Based Newborn Screening for Severe Combined Immunodeficiency Disease: A Systematic Review. Journal of Clinical Immunology. 2015;35(4):416-30. doi: 10.1007/s10875-015-0152-6.

125. Selimyan R, Gerstein RM, Ivanova I, Precht P, Subrahmanyam R, Perlot T, et al. Localized DNA demethylation at recombination intermediates during immunoglobulin heavy chain gene assembly.
PLoS biology. 2013;11(1):e1001475.

126. Guo C, Yoon HS, Franklin A, Jain S, Ebert A, Cheng H-L, et al. CTCF-binding elements mediate control of V (D) J recombination.
Nature. 2011;477(7365):424-30.

127. Bruniquel D, Schwartz RH. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process.
Nature Immunology. 2003;4:235. doi: 10.1038/ni887.

128. Maršál K, Persson PH, Larsen T, Lilja H, Selbing A, Sultan B. Intrauterine growth curves based on ultrasonically estimated foetal weights.
Acta paediatrica. 1996;85(7):843-8.

129. Stephansson O, Sandström A, Petersson G, Wikström AK, Cnattingius S. Prolonged second stage of labour, maternal infectious disease, urinary retention and other complications in the early postpartum period.
BJOG: An International Journal of Obstetrics & Gynaecology.
2016;123(4):608-16. doi: 10.1111/1471-0528.13287.

130. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells.
Journal of immunology (Baltimore, Md : 1950). 1984;133(1):157-65.
Epub 1984/07/01. PubMed PMID: 6586833.

131. Civin C, Trischmann T, Kadan N, Davis J, Noga S, Cohen K, et al. Highly purified CD34-positive cells reconstitute hematopoiesis.
Journal of Clinical Oncology. 1996;14(8):2224-33.

132. Marabita F, Almgren M, Lindholm ME, Ruhrmann S, Fagerström-Billai F, Jagodic M, et al. An evaluation of analysis pipelines for DNA methylation profiling using the Illumina HumanMethylation450 BeadChip platform.
Epigenetics. 2013;8(3):333-46. doi: 10.4161/epi.24008.

133. Karimi M, Johansson S, Stach D, Corcoran M, Grandér D, Schalling M, et al. LUMA (LUminometric Methylation Assay)—A high throughput method to the analysis of genomic DNA methylation. Experimental Cell Research.
2006;312(11):1989-95. doi: https://doi.org/10.1016/j.yexcr.2006.03.006.

134. Karimi M, Luttropp K, Ekström TJ. Global DNA methylation analysis using the luminometric methylation assay. Epigenetics Protocols. 2011:135-44.

135. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution.
Genomics. 2011;98(4):288-95.

136. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics. 2011;6(6):692-702.

137. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis.
BMC Bioinformatics. 2010;11:587. Epub 2010/12/02. doi: 10.1186/1471-2105-11-587. PubMed PMID: 21118553; PubMed Central PMCID: PMCPMC3012676.

138. Baker MW, Grossman WJ, Laessig RH, Hoffman GL, Brokopp CD, Kurtycz DF, et al. Development of a routine newborn screening protocol for severe combined immunodeficiency.
Journal of Allergy and Clinical Immunology. 2009;124(3):522-7.

139. Borte S, von Dobeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood. 2012;119(11):2552-5.
Epub 2011/12/02. doi: 10.1182/blood-2011-08-371021.
PubMed PMID: 22130802.

140. Keshi H, Sakamoto T, Kawai T, Ohtani K, Katoh T, Jang SJ, et al. Identification and Characterization of a Novel Human Collectin CL-K1.
Microbiology and immunology. 2006;50(12):1001-13.

141. Méndez-Lucas A, Hyroššová P, Novellasdemunt L, Viñals F, Perales JC. Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) is a pro-survival, endoplasmic reticulum (ER) stress response gene involved in tumor cell adaptation to nutrient availability.
Journal of Biological Chemistry. 2014;289(32):22090-102.

142. Henssen AG, Koche R, Zhuang J, Jiang E, Reed C, Eisenberg A, et al. PGBD5 promotes site-specific oncogenic mutations in human tumors.
Nat Genet. 2017;49(7):1005-14. doi: 10.1038/ng.3866
http://www.nature.com/ng/journal/v49/n7/abs/ng.3866.html#supplementary-information.

143. Lie BA, Todd JA, Pociot F, Nerup J, Akselsen HE, Joner G, et al. The predisposition to type 1 diabetes linked to the human leukocyte antigen complex includes at least one non-class II gene.
Am J Hum Genet. 1999;64(3):793-800. Epub 1999/03/03. doi:10.1086/302283.
PubMed PMID: 10053014; PubMed Central PMCID: PMCPMC1377797.

144. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nature biotechnology. 2010;28(5):495-501.
Epub 2010/05/04. doi: 10.1038/nbt.1630. PubMed PMID: 20436461; PubMed Central PMCID: PMCPMC4840234.

145. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. 2008;105(44):17046-9. Epub 2008/10/29. doi: 10.1073/pnas.0806560105. PubMed PMID: 18955703; PubMed Central PMCID: PMCPMC2579375.

146. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. Pediatric research. 2007;61(5 Pt 2):5R-10R. PubMed PMID: 17413851.

147. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Non-imprinted epigenetics in fetal and postnatal development and growth. Nestle Nutr Inst Workshop Ser. 2013;71:57-63. Epub 2013/03/19. doi: 10.1159/000342552. PubMed PMID: 23502139.

148. Gronlund MM, Nuutila J, Pelto L, Lilius EM, Isolauri E, Salminen S, et al. Mode of delivery directs the phagocyte functions of infants for the first 6 months of life. Clin Exp Immunol. 1999;116(3):521-6. Epub 1999/06/11. PubMed PMID: 10361245; PubMed Central PMCID: PMCPMC1905315.

149. Thilaganathan B, Meher-Homji N, Nicolaides KH. Labor: an immunologically beneficial process for the neonate. American journal of obstetrics and gynecology. 1994;171(5):1271-2. Epub 1994/11/01. PubMed PMID: 7977532.

150. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5. Epub 2013/11/15. doi: 10.1038/nature12726. PubMed PMID: 24226773; PubMed Central PMCID: PMCPMC3869884.

151. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50. Epub 2013/11/15. doi: 10.1038/nature12721. PubMed PMID: 24226770.

152. West CE, Jenmalm MC, Prescott SL. The gut microbiota and its role in the development of allergic disease: a wider perspective. Clinical & Experimental Allergy. 2015;45(1):43-53. doi: 10.1111/cea.12332.

153. Funkhouser LJ, Bordenstein SR. Mom knows best: the universality of maternal microbial transmission. PLoS Biol. 2013;11(8):e1001631. Epub 2013/08/27. doi: 10.1371/journal.pbio.1001631. PubMed PMID: 23976878; PubMed Central PMCID: PMCPMC3747981.

154. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy, intestinal colonization and human disease. Nat Rev Gastroenterol Hepatol. 2012;9(10):565-76. Epub 2012/08/15. doi: 10.1038/nrgastro.2012.144. PubMed PMID: 22890113.

155. Nemoda Z, Massart R, Suderman M, Hallett M, Li T, Coote M, et al. Maternal depression is associated with DNA methylation changes in cord blood T lymphocytes and adult hippocampi. Translational Psychiatry. 2015;5:e545. doi: 10.1038/tp.2015.32 https://www.nature.com/articles/tp201532#supplementary-information.

156. Vidal AC, Benjamin Neelon SE, Liu Y, Tuli AM, Fuemmeler BF, Hoyo C, et al. Maternal Stress, Preterm Birth, and DNA Methylation at Imprint Regulatory Sequences in Humans.
Genetics & Epigenetics. 2014;6:37-44. doi: 10.4137/GEG.S18067.
PubMed PMID: PMC4251062.

157. Fernando F, Keijser R, Henneman P, van der Kevie-Kersemaekers A-M, Mannens MM, van der Post J, et al. The idiopathic preterm delivery methylation profile in umbilical cord blood DNA. BMC genomics. 2015;16:736-.

158. Cruickshank MN, Oshlack A, Theda C, Davis PG, Martino D, Sheehan P, et al. Analysis of epigenetic changes in survivors of preterm birth reveals the effect of gestational age and evidence for a long term legacy.
Genome medicine. 2013;5(10):96.

159. Hillman SL, Finer S, Smart MC, Mathews C, Lowe R, Rakyan VK, et al. Novel DNA methylation profiles associated with key gene regulation and transcription pathways in blood and placenta of growth-restricted neonates. Epigenetics. 2015;10(1):50-61. doi: 10.4161/15592294.2014.989741.

160. McCullough LE, Mendez MA, Miller EE, Murtha AP, Murphy SK, Hoyo C. Associations between prenatal physical activity, birth weight, and DNA methylation at genomically imprinted domains in a multiethnic newborn cohort. Epigenetics. 2015;10(7):597-606. doi: 10.1080/15592294.2015.1045181.

161. Lillycrop K, Murray R, Cheong C, Teh AL, Clarke-Harris R, Barton S, et al. ANRIL Promoter DNA Methylation: A Perinatal Marker for Later Adiposity. EBioMedicine. 2017;19(Supplement C):60-72. doi: https://doi.org/10.1016/j.ebiom.2017.03.037.

162. van Mil NH, Steegers-Theunissen RPM, Bouwland-Both MI, Verbiest MMPJ, Rijlaarsdam J, Hofman A, et al. DNA methylation profiles at birth and child ADHD symptoms. Journal of Psychiatric Research. 2014;49(Supplement C):51-9. doi: https://doi.org/10.1016/j.jpsychires.2013.10.017.

163. Virani S, Dolinoy DC, Halubai S, Jones TR, Domino SE, Rozek LS, et al. Delivery type not associated with global methylation at birth. Clin Epigenetics. 2012;4(1):8. Epub 2012/06/12. doi: 10.1186/1868-7083-4-8. PubMed PMID: 22682523; PubMed Central PMCID: PMCPMC3404951.

164. Franz MB, Poterauer M, Elhenicky M, Stary S, Birner P, Vinatzer U, et al. Global and single gene DNA methylation in umbilical cord blood cells after elective caesarean: a pilot study. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2014;179(Supplement C):121-4. doi: https://doi.org/10.1016/j.ejogrb.2014.05.038.

165. Franz MB, Poterauer M, Elhenicky M, Stary S, Birner P, Vinatzer U, et al. Global and single gene DNA methylation in umbilical cord blood cells after elective caesarean: a pilot study. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2014;179:121-4.

166. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PloS one. 2012;7(7):e41361. Epub 2012/08/01. doi: 10.1371/journal.pone.0041361. PubMed PMID: 22848472; PubMed Central PMCID: PMCPMC3405143.

167. Socialstyrelsen. (2011) Indikationer för kejsarsnitt på moderns önskan. Rapport (2011: 09). Stockholm: Socialstyrelsen.

168. Yang H, Loutfy MR, Mayerhofer S, Shuen P. Factors affecting banking quality of umbilical cord blood for transplantation. Transfusion. 2011;51(2):284-92. Epub 2010/08/21. doi: 10.1111/j.1537-2995.2010.02826.x. PubMed PMID: 20723167.

169. Gasparoni A, Maccario R, Chirico G, Belloni C, Mingrat G, De Amici D, et al. Neonatal B lymphocyte subpopulations and method of delivery. Neonatology. 1992;61(3):137-41.

170. Herson VC, Block C, Eisenfeld LI, Maderazo E, Krause PJ. Effect of labor and delivery on neonatal polymorphonuclear leukocyte number and function. American journal of perinatology. 1992;9(04):285-8.

171. Steinborn A, Sohn C, Sayehli C, Baudendistel A, Hüwelmeier D, Solbach C, et al. Spontaneous labour at term is associated with fetal monocyte activation. Clinical and experimental immunology. 1999;117:147-52.

172. Koening J, Stegner J, Schmeck A, Saxonhounse M, Keningsberg L. Neonatal neutrophils with prolonged survival exhibit enhanced inflammatory and cytotoxic responsivness. Pediatr Res. 2005;57:424-9.

173. Marchini G, Berggren V, Djilali-Merzoug R, Hansson LO. The birth process initiates an acute phase reaction in the fetus-newborn infant. Acta paediatrica. 2000;89(9):1082-6.

174. 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 human development. 2005;81(4):387-92.

175. Sprent J, Tough DF. Lymphocyte life-span and memory. Science. 1994;265(5177):1395-401.

176. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GS, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: From birth to adulthood. Cytometry. 1996;26(1):8-15.

177. Lisse IM, Aaby P, Whittle H, Jensen H, Engelmann M, Christensen LB. T-lymphocyte subsets in West African children: impact of age, sex, and season. The Journal of pediatrics. 1997;130(1):77-85.

178. Klein SL, Flanagan KL. Sex differences in immune responses. Nature Reviews Immunology. 2016.

179. Pociot F, Nørgaard K, Hobolth N, Andersen O, Nerup J. A nationwide population-based study of the familial aggregation of Type 1 (insulin-dependent) diabetes mellitus in Denmark. Diabetologia. 1993;36(9):870-5. doi: 10.1007/bf00400364.

180. Almqvist C, Worm M, Leynaert B, for the working group of GALENWPG. Impact of gender on asthma in childhood and adolescence: a GA2LEN review. Allergy. 2008;63(1):47-57. doi: 10.1111/j.1398-9995.2007.01524.x.

181. Lee HS, Park Y-M, Han K, Pekler G, Lee S-S, Yoo S, et al. Sex-specific association between asthma and hypertension in nationally representative young Korean adults. Scientific Reports. 2017;7(1):15667. doi: 10.1038/s41598-017-15722-w.

182. Crea F, Battipaglia I, Andreotti F. Sex differences in mechanisms, presentation and management of ischaemic heart disease. Atherosclerosis. 2015;241(1):157-68.

183. Cremon C, Gargano L, Morselli-Labate AM, Santini D, Cogliandro RF, De Giorgio R, et al. Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. The American journal of gastroenterology. 2009;104(2):392-400.

184. Hunter TM, Boytsov NN, Zhang X, Schroeder K, Michaud K, Araujo AB. Prevalence of rheumatoid arthritis in the United States adult population in healthcare claims databases, 2004-2014. Rheumatology international. 2017;37(9):1551-7. Epub 2017/04/30. doi: 10.1007/s00296-017-3726-1. PubMed PMID: 28455559.

185. Dilokthornsakul P, Valuck RJ, Nair KV, Corboy JR, Allen RR, Campbell JD. Multiple sclerosis prevalence in the United States commercially insured population. Neurology. 2016;86(11):1014-21. doi: 10.1212/WNL.0000000000002469. PubMed PMID: PMC4799713.

186. Fischer J, Jung N, Robinson N, Lehmann C. Sex differences in immune responses to infectious diseases. Infection. 2015;43(4):399-403. doi: 10.1007/s15010-015-0791-9.

187. van Eijk LT, Dorresteijn MJ, Smits P, van der Hoeven JG, Netea MG, Pickkers P. Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers. Critical care medicine. 2007;35(6):1464-9.

188. Sharma AA, Jen R, Butler A, Lavoie PM. The developing human preterm neonatal immune system: a case for more research in this area. Clinical Immunology. 2012;145(1):61-8.

189. Kramer MS, Demissie K, Yang H, Platt RW, Sauvé R, Liston R, et al. The contribution of mild and moderate preterm birth to infant mortality. Jama. 2000;284(7):843-9.

190. Wirbelauer J, Thomas W, Rieger L, Speer CP. Intrauterine growth retardation in preterm infants≤ 32 weeks of gestation is associated with low white blood cell counts. American journal of perinatology. 2010;27(10):819-24.

191. Olearo E, Oberto M, Oggè G, Botta G, Pace C, Gaglioti P, et al. Thymic volume in healthy, small for gestational age and growth restricted fetuses. Prenatal diagnosis. 2012;32(7):662-7.

192. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia. 1993;36(1):62-7.
Epub 1993/01/01. PubMed PMID: 8436255.

193. Forsen T, Eriksson JG, Tuomilehto J, Osmond C, Barker DJ. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. Bmj. 1999;319(7222):1403-7.

194. Laitinen J, Pietilainen K, Wadsworth M, Sovio U, Jarvelin MR. Predictors of abdominal obesity among 31-y-old men and women born in Northern Finland in 1966. Eur J Clin Nutr. 2004;58(1):180-90.
Epub 2003/12/18. doi: 10.1038/sj.ejcn.1601765. PubMed PMID: 14679384.

195. Johansson S, Iliadou A, Bergvall N, Tuvemo T, Norman M, Cnattingius S. Risk of high blood pressure among young men increases with the degree of immaturity at birth. Circulation. 2005;112(22):3430-6. doi: 10.1161/CIRCULATIONAHA.105.540906. PubMed PMID: 16301344.

196. Olkhov-Mitsel E, Bapat B. Strategies for discovery and validation of methylated and hydroxymethylated DNA biomarkers.
Cancer Medicine. 2012;1(2):237-60. doi: 10.1002/cam4.22.
PubMed PMID: PMC3544446.

197. Birle A, Nebe CT, Hill S, Hartmann K, Poeschl J, Koch L. Neutrophil chemotaxis in cord blood of term and preterm neonates is reduced in preterm neonates and influenced by the mode of delivery and anaesthesia. PloS one. 2015;10(4):e0120341.

198. Eisler G, Hjertberg R, Lagercrantz H. Randomised controlled trial of effect of terbutaline before elective caesarean section on postnatal respiration and glucose homeostasis.
Archives of Disease in Childhood-Fetal and Neonatal Edition. 1999;80(2):F88-F92.

199. Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A, et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. Nature medicine. 2016;22(3):250.