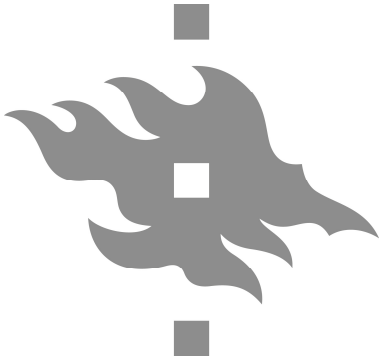

Effects of assisted reproductive technology on DNA methylation at the *IGF2/H19* gene locus and the phenotype of newborns

Master's Thesis

University of Helsinki

Department of Biosciences

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Tiivistelmä – Referat – Abstract

Assisted reproductive technology (ART) refers to treatments used for infertile couples to achieve pregnancy *in vitro*. The main technology of ART is *in vitro* fertilization (IVF), which may also include intracytoplasmic sperm injection (ICSI) and/or embryo cryopreservation and frozen embryo transfer (FET). ART treatments are well-accepted in Western countries and there is an increasing number of children being conceived in that way. Even though, majority of ART derived newborns appear healthy, they have been associated with increased risks of adverse perinatal outcomes, especially, alterations in birth size as well as higher frequencies of imprinting disorders and alterations in epigenetic modifications, such as in DNA methylation, of imprinted genes. Epigenetically regulated imprinted genes have crucial roles in fetal and placental growth during development and they are known to be affected by environmental factors. Since ART takes place in the early embryo in vulnerable time-period of epigenetic reprogramming, ART has been suggested to impact on epigenetic profiles of the embryo, consequently, affecting the phenotype of newborns, and therefore potentially causing long-term health effects.

This thesis aimed to study whether ART has effects on DNA methylation in the placenta and whether ART has effects on the phenotype of newborns. To study these effects, this thesis focused on the sixth binding sequence of CTCF (CTCF6) of *H19* ICR1 of the growth-related imprinted *IGF2/H19* gene locus. The aim was also to study whether the possible changes associate with the rs10732516 G/A polymorphism locating at CTCF6 of *H19* ICR1. DNA methylation levels of placental tissue as well as white blood cells in umbilical cord blood of ART derived, and spontaneously conceived newborns were explored by mass spectrometry-based Sequenom MassARRAY® EpiTYPER® method and traditional bisulfite sequencing. To study the effects of ART on the phenotype of newborns, the birth weight, length and head circumference of ART and control newborns were explored using international growth standards. Moreover, placental weights were compared.

The results of this thesis showed slightly, but consistently decreased DNA methylation levels at *H19* ICR1 in the paternal allele of ART derived placentas in rs10732516 patA/matG genotype, but not in patG/matA genotype. Thus, the results suggest that the changes in DNA methylation at *IGF2/H19* in the placenta are genotype-specific and associate with the rs10732516 polymorphism. Similar decreased methylation levels in the paternal allele of patA/matG genotype was not detected in white blood cells suggesting that the effects on DNA methylation levels are also cell type-specific. The effects of ART on the phenotype also associated with the rs10732516 polymorphism. Fresh embryo transfer derived newborns with A/A genotype were seen to have smaller birth weight than newborns with G/G genotype. Moreover, in A/A genotype, frozen embryo transfer derived newborns were demonstrated to be heavier and to have heavier placentas than fresh embryo transfer derived newborns.

The findings of this thesis suggest that ART has effects on DNA methylation in the placenta and on the phenotype of newborns, and the effects associate with the rs10732516 G/A polymorphism. This underlines the significance of the polymorphism when studying the effects of ART. However, further investigations are needed to confirm these findings and to discern whether the changes are due to the ART procedures or underlying infertility.

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Assisted reproductive technology, Imprinting, *IGF2/H19*, rs10732516 polymorphism, DNA methylation, Birth weight

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Tiivistelmä – Referat – Abstract

Hedelmöityshoidot ovat menetelmiä, jotka mahdollistavat raskauden alkamisen hedelmättömillä pariskunnilla *in vitro*. Yleisesti käytetyin hedelmöityhoitomenetelmä on koeputkihedelmöitys (*in vitro* fertilization, IVF), johon voi kuulua myös munasolun mikroinjektio ja/tai alkion pakastus ja pakastetun alkion siirto (frozen embryo trasfer, FET). Hedelmöityshoidot ovat yleisesti hyväksyttyjä menetelmiä länsimaissa ja hedelmöityshoitojen avulla syntyneiden lasten määrä on koko ajan nousussa. Vaikka hoitojen avulla syntyneet lapset ovat yleisesti ottaen terveitä, on ne yhdistetty erityisesti muutoksiin syntymäpainossa ja myös leimautumishäiriöiden kasvaneisiin määriin ja muutoksiin leimattujen geenien epigeneettisissä merkeissä kuten DNA:n metylaatiossa. Epigeneettisesti säädellyillä leimatuilla geeneillä on merkittävä rooli sikiön ja istukan kasvussa sikiönkehityksen aikana ja ympäristötekijöiden tiedetään vaikuttavan niihin. Koska hedelmöityshoidot sijoittuvat alkion epigeneettisen uudelleenohjelmoinnin ajanjaksolle, hoitojen on ajateltu vaikuttavan alkion epigeneettisiin profiileihin vaikuttaen vastasyntyneen fenotyyppiin ja täten mahdollisesti aiheuttaen pitkäaikaisia terveysvaikutuksia.

Tämän Pro gradu -tutkielman tarkoituksena oli tutkia vaikuttavatko hedelmöityshoidot istukan DNA:n metylaatioon ja onko hoidoilla myös vaikutusta vastasyntyneen fenotyyppiin. Näiden vaikutusten tutkimiseksi tutkielma keskittyi sikiön kasvuun liittyvään *IGF2/H19* geenilokukseen ja sen *H19* ICR1:n CTCF6 sekvenssiin. Tarkoituksena oli myös tutkia liittyvätkö muutokset CTCF6 sekvenssissä sijaitsevaan rs10732516 G/A polymorfiaan. Hedelmöityshoitojen avulla ja spontaanisti hedelmöitettyjen vastasyntyneiden DNA:n metylaatiotasot istukassa, ja myös napaveren valkosoluissa, tutkittiin massaspektrometriaan perustuvan Sequenomin MassARRAY® EpiTYPER® -menetelmän ja perinteisen bisulfiittisekvensoinnin avulla. Hedelmöityshoitojen vaikutusta vastasyntyneiden fenotyyppiin tutkittiin tarkastelemalla vastasyntyneiden syntymäpainoa, -pituutta ja päänympärystä kansainvälisten kasvukäyrien avulla. Myös istukoiden painoja vertailtiin.

Tämän tutkielman tulokset osoittivat hieman, mutta toistuvasti alentuneita DNA:n metylaatiotasoja rs10732516 patA/matG genotyypin *H19* ICR1:ssä istukassa. Muutoksia ei havaittu patG/matA genotyypissä, mikä osoittaa, että muutokset *IGF2/H19* lokuksen DNA:n metylaatiossa ovat genotyyppispesifisiä ja yhteydessä rs10732516 polymorfiaan. Samoja muutoksia patA/matG genotyypin paternaalisessa alleelissa ei myöskään havaittu napaveren valkosoluissa, mikä osoittaa, että vaikutukset riippuvat myös solutyypistä. Hedelmöityshoitojen vaikutukset fenotyyppiin havaittiin olevan myös yhteydessä rs10732516 polymorfiaan. Tuoreen alkion siirron avulla syntyneillä A/A genotyypin lapsilla havaittiin olevan pienempi syntymäpaino, kuin G/G genotyypin lapsilla. Lisäksi pakastetun alkion siirron avulla syntyneiden A/A genotyypin lapsien ja niiden istukoiden havaittiin olevan painavampia kuin tuoreen alkion siirron avulla syntyneiden lapsien ja istukoiden.

Tässä tutkielmassa hedelmöityshoitojen havaittiin vaikuttavan istukan DNA:n metylaatioon ja vastasyntyneen fenotyyppiin. Muutosten havaittiin olevan yhteydessä rs10732516 polymorfiaan, mikä korostaa kyseisen polymorfian merkittävyyttä tutkittaessa hedelmöityshoitojen vaikutuksia. Kuitenkin lisätutkimuksia tarvitaan tulosten varmistamiseen ja erottamaan johtuvatko muutokset hedelmöityshoitojen menettelytavoista vai taustalla olevasta hedelmättömyydestä.

Avainsanat – Nyckelord – Keywords	
Hedelmöityshoidot, Leimautuminen, *IGF2/H19*, rs10732516 polymorfia, DNA:n metylaatio, Syntymäpaino	
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List of Abbreviations

ART	Assisted reproductive technology
A^{vy}	Viable yellow agouti
BPA	Bisphenol A
CDC	Centers for Disease Control and Prevention
CpG	Cytosine-guanidine site
CTCF	Zinc-finger protein CCCTC-binding factor
CTCF6	The sixth binding sequence of CTCF
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DOHaD	Developmental origins of health and disease
ESHRE	European Society of Human Reproduction and Embryology
FET	Frozen embryo transfer
FIMM	Institute for Molecular Medicine Finland
ICMART	International Committee for monitoring Assisted Reproduction Technologies
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IGF2	Insulin growth factor 2
IUGR	Intrauterine growth restriction
IVF	*In vitro* fertilization
LGA	Large for gestation age
LincRNA	Large intergenic non-coding RNA
LINE	Long interspersed nuclear element
LOI	Loss of imprinting
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-light
ncRNA	Non-coding RNA
PGC	Primordial germ cells
SAM	S-adenosylmethionine
SD	Standard deviation
SGA	Small for gestation age
SNP	Single nucleotide polymorphism
SINE	Short interspersed nuclear element
WBC	White blood cell
WHO	The World Health Organization

1 Introduction

Assisted reproductive technology (ART) refers to treatments used for infertile couples to achieve pregnancy by handling human oocytes and sperm, or embryos, outside of the body, *in vitro* (Zegers-Hochschild et al., 2009). ART treatments are well-accepted and common procedures in Western countries and there is an increasing number of children being conceived in that way (The European IVF-monitoring Consortium et al, 2013). Even thought, majority of the newborns conceived by these treatments appear heathy, ART has been associated with increased risks of adverse perinatal outcomes, especially, alterations in birth size: fresh embryo transfer derived newborns have been associated with low birth weight (Jackson et al., 2004) and, by contrast, frozen embryo transfer derived (FET) newborns have been associated with increased birth weight (Pelkonen et al., 2010; Wennerholm et al., 2013; Maheshwari et al. 2018). In addition, ART children have been associated with higher frequencies of imprinting disorders (Lazaraviciute, Kauser, Bhattacharya, Haggarty, & Bhattacharya, 2014; Ludwig et al., 2005; Maher et al., 2003) and alterations in epigenetic modifications, such as in DNA methylation, of imprinted genes (Castillo-Fernandez et al., 2017; Katari et al., 2009; Loke, Galati, Saffery, & Craig, 2015; Nelissen et al., 2013a).

Epigenetically regulated imprinted genes have crucial roles in fetal and placental growth during gestation and they are known to be affected by environmental factors (Plasschaert & Bartolomei, 2014). ART takes place in the early embryo in vulnerable time-period of epigenetic reprogramming when epigenetic marks, including DNA methylation, are erased and re-established. Since ART has been associated with imprinting disorders and alterations in the newborn size and due to the important role of imprinted genes in the development and growth of the embryo, there has been growing interest in DNA methylation variation in imprinted genes of ART newborns. ART has been suggested to impact on epigenetic profiles of the embryo, consequently, affecting the phenotype of newborns, and therefore potentially causing long-term health effects.

Owing to that, the aim of this thesis was to study the effect of ART on DNA methylation of the growth-related *Insulin growth factor 2* (*IGF2)/H19* imprinted gene locus in the placenta, and in white blood cells (WBCs) in umbilical cord blood, as well as the effect of ART on the phenotype of newborns.

1.1 ART – Assisted reproductive technology

Assisted reproductive technology (ART) refers to a wide range of treatments or procedures that can be used to achieve pregnancy by handling human oocytes and sperm, or embryos, outside of the body, *in vitro* (Zegers-Hochschild et al., 2009). The main technology of ART is *in vitro* fertilization (IVF), which may also include intracytoplasmic sperm injection (ICSI) and/or embryo cryopreservation and frozen embryo transfer (FET). Other examples of ARTs are gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, egg and embryo donation and gestational surrogacy. Procedures in which only sperm is handled, such as intrauterine insemination, or treatments where only medicine is used to stimulate women's oocyte production (ovarian stimulation) are not considered as ART (Centers for Disease Control and Prevention, 2017).

Assisted reproductive technologies are primarily used due to infertility of either woman or men, but also due to genetic (preimplantation genetic diagnosis) (Soini et al., 2006) or other diseases such as HIV (Ohl et al., 2003). Infertility is a disorder of the reproductive system, which results in an inability to conceive after trying 12 months (Zegers-Hochschild et al., 2009). It is a significant health problem affecting over 48 million couples worldwide (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). The causes of infertility vary, half being associated with male and half with female factors, however, sometimes the cause cannot be identified. The most common causes of infertility in women are ovulation disorders, uterine or cervical abnormalities, fallopian tube damage or blockage or endometriosis (Healy, Trounson, & Andersen, 1994). Age is a major influencer of women infertility. Fertility of women starts to decrease after 30 years old and declines rapidly after 35 years as women's stock of eggs starts to decrease up to the menopause (Healy et al., 1994). Consequently, the success rate of IVF treatment decreases as well (The European IVF-monitoring Consortium et al., 2013). The causes of infertility of men include abnormal sperm production (oligozoospermia and azoospermia), motility (asthenozoospermia) or morphology (teratospermia) and problems with the delivery of sperm (Kumar & Singh, 2015). Genetic factors can cause infertility in both woman and men, but they are more commonly associated with male infertility (Poongothai, Gopenath, & Manonayaki, 2009). In both, woman and men, several lifestyle factors, such as smoking, alcohol and exercise may affect fertility (Healy et al., 1994).

Louise Brown was the first child successfully fertilized using IVF in England in 1978 (Steptoe & Edwards, 1978). Nowadays, ART treatments are well-accepted and common procedures

in Western countries and there are an increasing number of ART treatment cycles and children born conceived using ART procedures. According to ART data of 38 countries in Europe, collected by European Society of Human Reproduction and Embryology (ESHRE) and the International Committee for monitoring Assisted Reproduction Technologies (ICMART), there were more than 685 000 ART cycles reported in 2013 (The European IVF-monitoring Consortium et al., 2013). According to Centers for Disease Control and Prevention (CDC), in the United States, every year approximately 1.6% of all infants are conceived using ART and there were over 200 000 ART treatment cycles reported in 2015 (Centers for Disease Control and Prevention et al., 2017). In Finland, the number of ART cycles has also increased over the last decade and over 9000 ART treatments are started annually (Figure 1) (Terveyden ja hyvinvoinnin laitos, 2017).

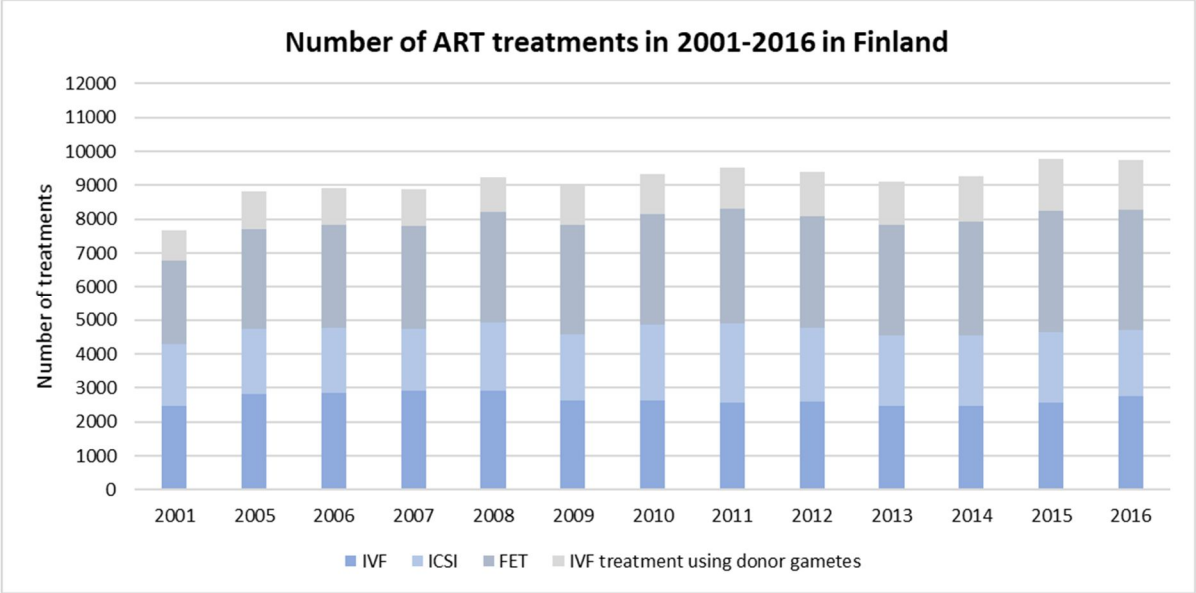


Figure 1. Number of started ART treatments in 2001–2016 in Finland. The number of ART cycles has increased over the last decade and over 9000 ART cycles are started annually. Data for the year 2016 were preliminary. Modified from Terveyden ja hyvinvoinnin laitos (2017).

Success rates of ART vary depending on the type of ART technique and the patient's age, infertility diagnosis, and previous births, miscarriages and fertility treatments (Centers for Disease Control and Prevention et al., 2017). Women often need to undergo several treatments and not all successful pregnancies, resulted by ART, lead to delivery of a live infant. According to ESHRE and ICMART, in Europe in 2013, the delivery rates per aspiration using IVF treatment was 22.2% and using ICSI treatment 20.1%. The delivery rate per thawing for frozen embryo replacement was 18% (The European IVF-monitoring Consortium et al., 2013). According to CDC, in 2015 in the United States, the delivery rate

per fresh cycle using ART was slightly higher than in Europe: 24% (Centers for Disease Control and Prevention et al., 2017).

Different countries have different regulations for the use of ART and fertility treatments. Almost every country in EU has own legislation. However, in EU the patients have freedom of movement to another country for the treatment (European Society of Human Reproduction and Embryology, 2017). The main differences in the regulations are related to embryo selection by genetic screening, embryo freezing, and transfer as well as egg donation. In the United States, there is no national legislation for ART and the regulation is governed by professional guidelines and federal law of general regulation of medical practice and laboratory conditions (European Society of Human Reproduction and Embryology, 2017). In Finland, the Act on Assisted Fertility Treatments (1237/2006) entered into force in 2007 and governs the usage of gametes and embryos to achieve pregnancy as well as the donation and storage of gametes and embryos for treatments.

1.1.1 IVF – *In vitro* fertilization

In *in vitro* fertilization (IVF), egg and sperm cells are fertilized outside of the body in a laboratory environment and it is defined by ICMART and the World Health Organization (WHO) as “an ART procedure that involves extracorporeal fertilization” (Zegers-Hochschild et al., 2009). It is the most used ART procedure worldwide as 6.5 million IVF babies have been born since Louise Brown. It can be used almost in any cases of infertility diagnosis and the eggs, sperm or embryos can be also donated (Tiitinen, 2017b).

The IVF procedure starts with ovarian stimulation protocol, in which different combinations of hormones and medicines are given to stimulate the oocyte production generating several oocytes at a time (Pacchiarotti et al., 2016). The eggs are collected using oocyte retrieval, in which a needle is passed through a vaginal wall into an ovarian follicle under ultrasound guidance and the follicle fluid containing the oocytes is aspirated (Tiitinen, 2017b). The mature oocytes are identified and passed into a culture media. For fertilization, an average of 200 000 sperm cells per egg is added to the culture media (Tiitinen, 2017b). The day after, the fertilized eggs are revised, and the culturing is continued at least for two days. After the two days the embryos can be already transferred into the uterus or, more frequently, the culturing is continued until days five or six. In this stage, the embryo is called a blastocyst (Tiitinen, 2017b). When culturing the embryos to the blastocyst stage, it is possible to select the best quality embryos, which increases the pregnancy and implantation success rates and

enables using fewer embryos (1-2) for transfer into the uterus, hence reducing multiple pregnancy risk (Gardner et al., 1998).

Intracytoplasmic sperm injection (ICSI) is a procedure in which microinjection is used to transfer a single sperm cell into the oocyte cytoplasm. Otherwise, the treatment is carried out as IVF (Zegers-Hochschild et al., 2009). The method has been in use since the early 1990s and it is used especially when there is not enough sperm, or it is poorly moving (Palermo, Joris, Devroey, & Van Steirteghem, 1992). In fact, over the last years, ICSI has developed to be the most important treatment for male factor infertility (Tiitinen, 2017b).

After IVF treatment, approximately 60–70% cycles result in several embryos (Tiitinen, 2017a). Since usually only one embryo is used for transfer, the additional embryos can be chosen to cryopreserve and thaw for another attempt or store for a later use. This procedure is called frozen embryo transfer (FET) (Zegers-Hochschild et al., 2009). As a benefit of FET, it is possible to preserve the good quality embryos and use only a single embryo for transfer and, hence, avoid multi-gestation (Aflatoonian et al., 2016). In addition, FET is considered to have similar or even higher pregnancy and delivery rates when compared to freshly collected embryos. One reason for this could be that FET enables the embryo transfer in a natural cycle without ovarian stimulation, which increases implantation success. FET is also beneficial in the case of the ovarian hyperstimulation syndrome (Aflatoonian et al., 2016) in which ovarian response to ovarian stimulation hormones and medicines is excessive (The Practice Committee of the American Society for Reproductive Medicine, 2008).

1.1.2 Perinatal outcomes of ART

Majority of the newborns conceived by *in vitro* fertilization with or without intracytoplasmic sperm injection, using fresh embryos, appear healthy. However, in a number of studies, when compared to spontaneously conceived newborns, IVF and IVF/ICSI newborns have been associated with problems in pregnancies and increased risks of adverse perinatal outcomes, especially intrauterine growth restriction (IUGR) resulting in newborns with low birth weight and newborns small for gestational age (SGA) (Doyle et al., 1992; Helmerhorst, Perquin, Donker, & Keirse, 2004; Jackson et al., 2004; Malchau, Lof, Henningsen, Nyboe, & Pinborg, 2014). IUGR newborns are SGA if they are -2 standard deviations (SDs) below the mean birth weight for gestational age (Clayton et al., 2007). On the other hand, frozen embryo transfer has been seen to increase the risk of large for gestational age (LGA) infants (Pelkonen et al., 2010; Wennerholm et al., 2013; Maheshwari et al., 2018). Newborns large

for gestational age (LGA) have +2 SDs over the mean birth weight for gestational age (Clayton et al., 2007). IVF and IVF/ICSI have also been associated with multiple-birth deliveries (Thurin et al., 2004) preterm births (Doyle et al., 1992; Jackson et al., 2004) and birth defects (Hansen, Kurinczuk, Milne, de Klerk, & Bower, 2013). Some studies have associated IVF and IVF/ICSI with increased risk for placental complications: pregnancies conceived by IVF have been seen to result in increased placental weight and placental weight/birthweight ratio compared to spontaneous pregnancies (Haavaldsen, Tanbo, & Eskild, 2011).

The reason for the adverse perinatal outcomes has suggested being, for example, *in vitro* embryo culture conditions and ovarian stimulation used in IVF treatment (El Hajj & Haaf, 2013) There is an increasing evidence that the type of culture medium used *in vitro* culture of the embryos affects fetal development, especially causing low birthweight of IVF newborns (Dumoulin et al., 2010; Nelissen et al., 2012, 2013b). On the other hand, long *in vitro* culture (from day 2 to days 5-6) has been seen to increase the risk for large for gestational age infants (Mäkinen, Söderström-Anttila, Vainio, Suikkari, & Tuuri, 2013).

However, the results are inconsistent and the reason for these adverse perinatal outcomes have suggested not to be the IVF procedure itself, but rather the underlying parental infertility, since infertility has also been associated with pregnancy complications, birth defects and lower birth weights (Basso & Baird, 2003; Luke et al., 2016; Palomba, Santagni, Gibbins, La Sala, & Silver, 2016; Seggers et al., 2015). In addition, the reason for the adverse perinatal outcomes has also suggested being multiple pregnancies or older parental age, which both are linked to IVF births.

1.2 Epigenetics

The term “epigenetics”, introduced by Conrad H. Waddington in 1942, was originally defined as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Goldberg, Allis, & Bernstein, 2007). The meaning of epigenetics changed during the following 50 years, and in 1996 Riggs et al. (1996) defined the epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence”. Epigenetic mechanisms are important during early development when they enable the proper formation and maintenance of specific cell lineages and tissue types by adapting to

environmental signals and controlling gene expression (Das, 2016). There are several phenomena in which epigenetics are involved in mammalian cells: epigenetic reprogramming in embryogenesis and gametogenesis, tissue-specific gene expression, genomic imprinting, X chromosome inactivation, genome stability and DNA heterochromatinization (Jirtle & Skinner, 2007). Changes in the epigenome are required for a normal embryo development, but disruption of the mechanisms can cause epigenetic alterations and abnormal gene expression. Epigenetic changes have been associated with several diseases, including cancer, autoimmune diseases and neurodegenerative and psychological disorders (Moosavi & Ardekani, 2016). The exposure to environmental factors *in utero* or in later life is suggested to affect the establishment and maintenance of epigenetic modifications. These environmental factors include chemicals, diet, exercise, stress, obesity, tobacco smoking and alcohol consumption (Alegría-Torres, Baccarelli, & Bollati, 2011).

1.2.1 Epigenetic modifications

Three main epigenetic modifications are well characterized: modifications of histone proteins, non-coding RNAs (ncRNAs) and, most significantly, DNA methylation (Weinhold, 2006). These mechanisms do not function alone, but rather work together and cross-regulate each other (Cedar & Bergman, 2009).

The histone modifications alter gene expression by remodeling the chromatin structure via histone post-translational chemical modifications, histone binding chromatin factors and by replacement of specific histones by histone variants (Bannister & Kouzarides, 2011). How chromatin is organized in its three-dimensional structure affects how the genes can be expressed. The more open chromatin allows transcription factors to bind on promoters and different interactions between regulatory DNA domains enabling gene expression. By contrast, more condensed chromatin silences the genes (Strachan, Goodship, & Chinnery, 2014, p. 161–162). In chromatin, the basic unit is nucleosome, in which ~147 bp of DNA is wrapped around two molecules of each canonical histone H2A, H2B, H3 and H4 (core histones) (Strachan et al., 2014, p. 161–162). Histones have positively charged histone N-terminal tails that can be chemically modified such as acetylated, phosphorylated or methylated post-translationally at specific residues affecting the chromatin structure and consequently the gene expression (Strahl & Allis, 2000). The enzymes that add histone modifications (such as histone acetyltransferases and methyltransferases) are often defined as writers, and the enzymes that remove the modifications (such as histone deacetylases and demethylases) as erasers (Gillette & Hill, 2015). Moreover, the post-translational histone

modifications in specific amino acids can be recognized by nonhistone proteins, also defined as readers, that further recruit chromatin modeling proteins, or chromatin factors, that alter the chromatin structure (Gillette & Hill, 2015; Smith & Peterson, 2005). The chromatin structure and function can be also altered by replacement of core canonical histones by noncanonical histone variants, mainly histone classes H2A and H3 (Henikoff & Ahmad, 2005). These variants typically differ from each other by few amino acids affecting the histone incorporation into chromatin, assembly of overall nucleosome structure and possibly the post-translational histone modifications.

NcRNAs regulate the gene function at transcriptional and post-transcriptional level. NcRNAs that are involved in epigenetic regulation include long ncRNAs and short ncRNAs, such as microRNAs, short-interfering RNAs and piwi-interacting RNAs (Costa, 2008; Cao, 2014). These ncRNAs, involved in epigenetic regulation, have roles in genomic imprinting, DNA heterochromatinization, X chromosome inactivation, histone modification and DNA methylation directing as well as transcriptional gene regulation.

Perhaps the most significant and the most extensively studied epigenetic modification is the DNA methylation. DNA methylation is a mechanism in which a methyl group (CH_3) is covalently attached from S-adenosylmethionine (SAM) to the 5-carbon of the cytosine ring resulting in 5-methylcytosine (5-meC). DNA methylation is associated with a repressed chromatin state and transcription inhibition. It plays a crucial role in embryonic development, chromatin structure, X chromosome inactivation, genomic imprinting and genome stability by controlling repetitive and transposable elements (such as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs)) (Robertson, 2005). DNA methylation can occur at any cytosine of the genome, but in somatic cells, most of the DNA methylation occurs in the context of cytosine-guanidine (CpG) sites (Lister et al., 2009). From 70% to 80% of all CpG sites in the genome are methylated, while CpG islands, CpG-rich regions positioned near gene promoters, generally remain unmethylated allowing gene expression (Bird, 2002). Methylation in the CpG island blocks the enhancer elements and promoters from transcription factors, preventing gene expression (Robertson, 2005). This kind of gene silencing is involved, for example, in genomic imprinting (Bird, 2002). Transfer of the methyl groups is carried out by an enzyme family of DNA methyltransferases (DNMTs). Different DNMTs are responsible for the establishment and maintenance of DNA methylation patterns (Jin, Li, & Robertson, 2011). DNMT3A and DNMT3B are known as de novo DNMTs as they transfer the initial methylation patterns during development. Maintenance methyltransferase DNMT1 enables the inheritance of DNA methylation through

cell division. DNMT1 is known to maintain the DNA methylation patterns by methylating hemimethylated DNA, during cell division and replication, using the existing DNA strand as a template (Jin et al., 2011). DNA demethylation, which is an important process in epigenetic reprogramming in the early embryo, can occur actively by an enzymatic process or passively during replication in the absence of maintenance methylation (Kohli & Zhang, 2013).

Abnormal changes in these epigenetic modifications, leading to aberrant gene expression, are called epimutations. The underlying cause of the epimutation may be a mutation in the DNA sequence of a gene that encodes a protein or RNA, such as histone or DNA modifying enzymes, that control epigenetic modifications (Horsthemke, 2006). Moreover, it is known that single nucleotide polymorphisms (SNPs) may affect the methylation levels of nearby CpG sites (Feil & Fraga, 2012). Epimutations can also arise due to environmental exposures without any change to the DNA sequence. The environmental exposures that affect the epigenetic modifications are further introduced in section 1.2.3 Environmental epigenetics.

1.2.2 Epigenetic reprogramming

In epigenetic reprogramming, epigenetic marks, especially DNA methylation, are erased and re-established in the early embryo. It is an important event for the developing embryo to re-acquire the totipotency of the cells and initiate the formation of different cell lines. The reprogramming occurs in two phases: during gametogenesis in the primordial germ cells (PGCs), which eventually give rise to gametes, and during embryogenesis in the preimplantation embryo (Figure 2) (Santos & Dean, 2004). The reprogramming occurs during gametogenesis when epigenetic marks are erased in PGCs in the developing embryo (Santos & Dean, 2004). The PGCs migrate into the genital ridge and undergo global DNA demethylation, restoring the totipotency. Thereafter, *de novo* methylation allows the epigenetic patterns to be re-established in male germ cells during spermatogenesis and in female germ cells during oocytes growth. In embryogenesis, the reprogramming occurs after fertilization and formation of the zygote when global DNA demethylation, by active mechanisms in a paternal and passive mechanics in a maternal genome, begins and continues until the blastocyst stage (Santos & Dean, 2004). After the fifth cell cycle, genome-wide *de novo* methylation, from which CpG islands are protected, occurs congruent with initial differentiation steps, leading to the formation of individual cell types. The first two cell lines result in the hypermethylated inner cell mass, which gives rise to all adult tissues and the hypomethylated trophectoderm, which forms the extra-embryonic tissues of the placenta.

This imbalance in the DNA methylation between the embryo and the placenta continues throughout gestation.

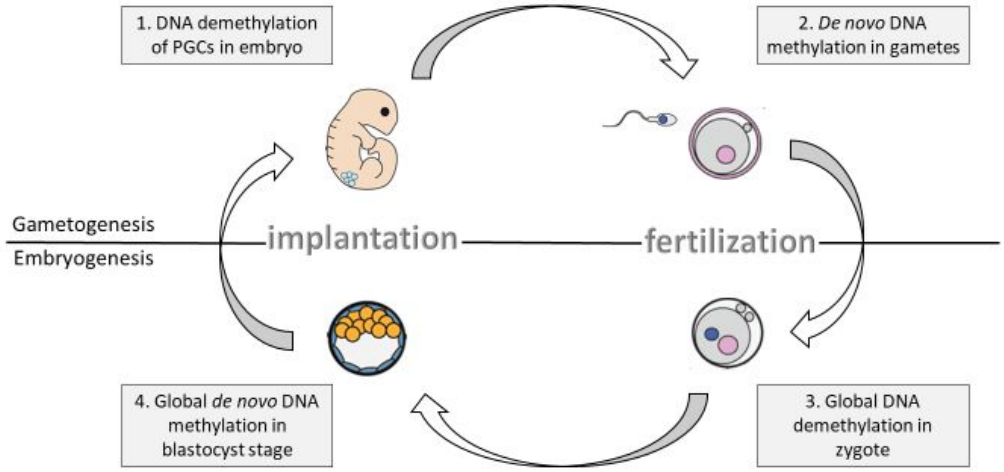


Figure 2. Epigenetic reprogramming. In gametogenesis, the epigenetic reprogramming occurs when PGCs in developing embryo migrate into the genital ridge and undergo global DNA demethylation (1). *De novo* methylation allows the epigenetic patterns to be re-established in male germ cells during spermatogenesis and in fame germ cells during oocyte growth (2). In embryogenesis, the epigenetic reprogramming occurs after fertilization and formation of the zygote when global DNA demethylation, by active mechanisms in a paternal and passive mechanics in a maternal genome, begins and continues until the blastocyst stage (3). After the fifth cell cycle, genome-wide *de novo* methylation occurs leading to the formation of individual cell types (4).

The reprogramming in the early embryo underlines the importance of *in utero* conditions during early development. Human epidemiological studies have suggested that environmental factors during development influence the susceptibility to develop diseases, such as cancer, heart disease, obesity or diabetes, in later life (Jirtle & Skinner, 2007). These fetal effects on adult diseases are generally defined as “developmental origins of health and disease” (DOHaD) (Barker, 2004). Previously, the focus has been on the genome, but nowadays there is increasing evidence that epigenetics is also involved (Jirtle & Skinner, 2007). Early embryogenesis is a critical time for the establishment of epigenetic marks, and since the patterns are mitotically inherited in somatic cells, alterations may have long-time health effects. The environment in which the embryo develops has been seen to affect the epigenome, such as DNA methylation patterns, having associations with later-onset disease. Some studies also suggest that some of these changes may be transgenerationally inherited when occurring in germ cells (Jirtle & Skinner, 2007).

1.2.3 Environmental epigenetics

The connection between environmental exposures, epigenetics and diseases was already seen in children who had a higher risk of having obesity, heart diseases and diabetes in adulthood after maternal exposure to famine during the “Hunger winter” in 1944–45 in the Netherlands (Painter, Roseboom, & Bleker, 2005). Heijmans et al. (2008) found an association between periconceptional famine exposure and decreased DNA methylation in the *IGF2* imprinted gene. In further studies, DNA methylation changes were also detected in other imprinted genes (Tobi et al., 2009). Since then, several environmental exposures, such as chemicals and nutrition components, during embryo development have been seen to cause epigenetic alterations leading to diseases. One example of the chemicals is bisphenol A (BPA), a plastic additive, that has now been displayed to cause several health risks (Halden, 2010). Susiarjo et al. (2013) illustrated that maternal BPA exposure during early embryonic development significantly altered the expression and methylation of imprinted genes in the mouse. Furthermore, prenatal exposure to dietary methyl-donor supplementation with folic acid, B vitamins, betaine, and choline, have been linked to DNA methylation changes (Anderson, Sant, & Dolinoy, 2012). These micronutrients are required for the one-carbon metabolism pathway that affects the availability of DNA and histone methyl-donor S-adenosylmethionine (SAM) (Anderson et al., 2012)

The effects of environmental exposures on epigenome have also been illustrated in studies with the viable yellow agouti (A^{vy}) mouse model, which can be used as an epigenetic biosensor for environmental factors. In the agouti mouse model, DNA methylation at metastable epiallele, A^{vy} locus, correlates with the expression of the gene leading to a range of coat colors from yellow to pseudoagouti (Morgan, Sutherland, Martin, & Whitelaw, 1999). DNA methylation at A^{vy} in the mouse offspring has been seen to increase after maternal diet supplemented with methyl donors leading to a pseudoagouti coat color (Waterland & Jirtle, 2003). The same effect on A^{vy} epiallele and phenotype has also been seen in offspring after *in utero* exposure to maternal ethanol consumption (Kaminen-Ahola et al., 2010). On the contrary, maternal exposure to BPA has been seen to decrease the DNA methylation level at the A^{vy} epiallele leading to yellow coat color of the offspring, along with obesity and diabetes (Dolinoy, Huang, & Jirtle, 2007).

The early embryonic development around the epigenetic reprogramming period has been seen to be particularly sensitive to the environmental factors. Studies on the Hunger winter demonstrated that the DNA methylation changes of imprinted genes were only specific to the

periconceptional famine exposure and not associated to famine exposure in later stages of gestation (Heijmans et al., 2008; Painter et al., 2005). Furthermore, in Susiarjo et al. (2013) study, the effects of BPA exposure on DNA methylation of imprinted genes were only specific to the earliest stages of embryonic development around the epigenetic reprogramming period.

1.3 Imprinting

Mammals have two copies of each chromosome: one from the mother and one from the father. Genes from the paternal and maternal alleles are expressed or repressed biallelically. However, in a small part of our genes, only one of the two alleles is expressed and the other allele is consistently silenced according to parent-of-origin. These genes are known as imprinted genes, and the phenomena behind it as genomic imprinting (Peters, 2014; Plasschaert & Bartolomei, 2014; Reik & Walter, 2001). To date, there are over 150 imprinted genes found in humans and many of these genes are conserved in mammals ("Imprinted Genes: by Species"). The fact that a subset of paternally expressed genes enhance fetal growth, as maternally expressed suppresses this growth, has led to a hypothesis of the evolution of imprinting. The genetic conflict theory, also known as kinship theory, suggests that there is a conflict between the interests of paternal and maternal genes (Moore & Haig, 1991). Paternally expressed genes are proposed to extract resources via the placenta to promote the growth and the fitness of the fetus at the expense of the mother, whereas maternally expressed genes are proposed to limit nutrient transfer to ensure the mother's health and future reproductive success (Moore & Haig, 1991). The expression of imprinted genes varies between tissues and developmental stages. During gestation, imprinted genes have crucial roles in fetal and placental growth, and in cell type-specific functions, especially in the brain (Plasschaert & Bartolomei, 2014). The most extensively studied example is the *IGF2/H19* imprinted gene locus that regulates the prenatal growth of the fetus in the placenta. Imprinted genes also have important roles after birth in behavior, such as maternal care, metabolism and the maintenance and function of adult stem cells (Plasschaert & Bartolomei, 2014).

Imprinted genes are epigenetically regulated, typically rich in CpG islands and found in clusters of 3–12 genes (Plasschaert & Bartolomei, 2014; Reik & Walter, 2001). DNA methylation has a significant role in the establishment and maintenance of imprinting. Imprinted gene clusters are under the control of *cis*-acting imprinting control region (ICR),

which is differentially methylated in parent-of-origin manner and controls gene expression within imprinted regions over large distances. The promoter of the imprinted gene typically contains a CpG island, which is also often differentially methylated, the methylated allele being repressed and the unmethylated allele being expressed. These regions are called differentially methylated regions (DMRs) (Plasschaert & Bartolomei, 2014; Reik & Walter, 2001). The ncRNA model and insulator model are two well-defined regulation mechanisms of imprinted genes (Plasschaert & Bartolomei, 2014). The ncRNA model, employed for example at *Igf2r/Airn* and *Kcnq1/Kcnq1ot1* loci, utilizes an antisense transcript, long ncRNA, which act as a silencer for the neighboring genes of the imprinted gene (Plasschaert & Bartolomei, 2014). The expression of the ncRNA is regulated by ICR in its CpG-rich promoter. In the paternal allele, the ICR is unmethylated, allowing gene expression of the ncRNA, whereas in the maternal allele the ICR is methylated, allowing the repression of the ncRNA and expression of the neighboring genes. One example of the insulator model is the regulation of *Igf2/H19* gene locus, which utilizes zinc-finger protein CCCTC-binding factors and their binding to ICR. This model is further described in section 1.4 *IGF2/H19* imprinted gene locus.

Imprinting marks follow a life cycle (Figure 3) in which they are erased, re-established and maintained in cells, which is crucial for proper parent-of-origin gene expression (Plasschaert & Bartolomei, 2014; Reik & Walter, 2001). During the epigenetic reprogramming, all the epigenetic marks, including DNA methylation and imprinting marks, are erased in primordial germ cells in the developing embryo. After erasure, the imprinting marks are established by DNA methylation in ICRs by *de novo* methyltransferases in germ lines in a sex-specific manner. In male germ cells, the establishment occurs prenatally during spermatogenesis and in female germ cells postnatally during oocyte growth. After fertilization, these imprinting marks are retained, despite the global DNA demethylation in preimplantation embryo and protected from the *de novo* methylation in the postimplantation embryo, and further maintained in somatic cells throughout adulthood.

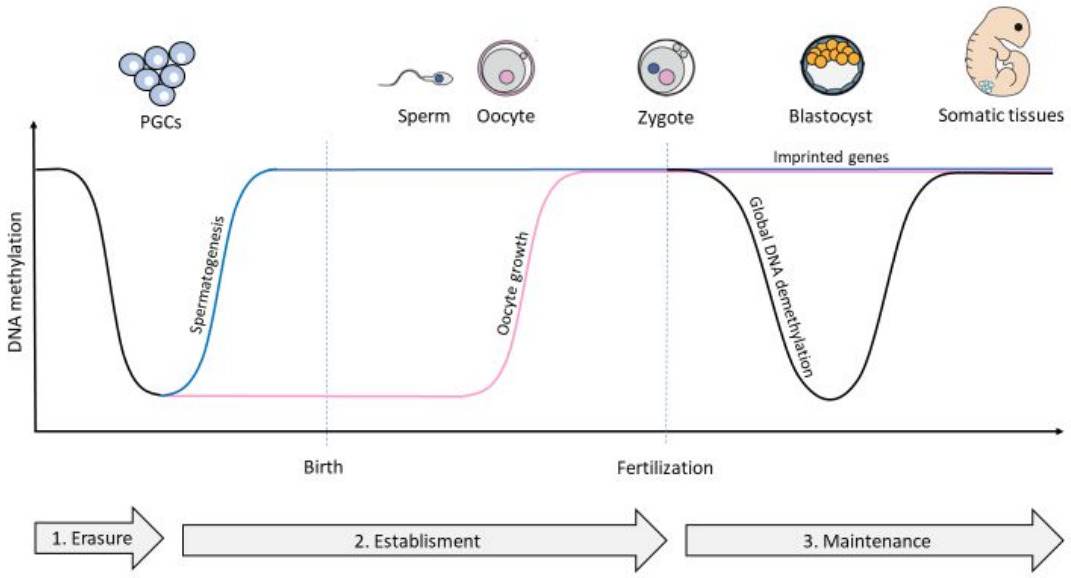


Figure 3. The life cycle of imprinting marks. During epigenetic reprogramming, all epigenetic marks, including imprinting marks, are erased in primordial germ cells in the developing embryo (1). After erasure, the imprinting marks are re-established in germ lines in a sex-specific manner. In male germ cells the establishment occurs prenatally during spermatogenesis (blue line) and in female germ cells postnatally during oocyte growth (pink line) (2). After fertilization, these imprinting marks are retained, despite the global DNA demethylation in the preimplantation embryo and maintained in somatic tissues throughout adulthood (3).

1.3.1 Imprinting errors

Since many imprinted genes have significant roles in fetal growth and development, but also in adult tissue, the dysregulation of these genes may result in disease. Disrupted imprinting has been implicated in several diseases, including intrauterine growth restriction (IUGR), obesity, diabetes, cancer, such as in Wilms' tumor, and imprinting disorders, including the growth disorders Beckwith-Wiedemann and Silver-Russell syndromes and the neuro-developmental disorders Prader-Willi and Angelman syndromes (Peters, 2014; Robertson, 2005). The disease may arise due to the loss or gain of imprinted gene expression, and therefore loss of imprinting (LOI). One mechanism is uniparental disomy, which occurs when both chromosomes originate either from the mother or the father and the chromosome includes imprinted genes important for development (Peters, 2014). Moreover, epimutations or mutations in a DNA sequence in imprinted genes may result in abnormal imprinted gene expression and LOI (Peters, 2014). DNA methylation changes are often the underlying cause of the aberrant imprinted gene expression in several diseases (Robertson, 2005). DNA methylation, which has a crucial role in the monoallelic expression of imprinted genes, is vulnerable to different environmental factors around epigenetic reprogramming and early

embryogenesis. Prenatal exposure to maternal malnutrition (Heijmans et al., 2008), diet (Feil & Fraga, 2012), tobacco smoking (Breton *et* al., 2009) and alcohol consumption (Marjonen, Kahila, & Kaminen-Ahola, 2017), as well as assisted reproductive technology (Castillo-Fernandez et al., 2017; Katari et al., 2009; Loke et al., 2015; Nelissen et al., 2013a), has been linked to DNA methylation alterations in imprinted genes.

1.3.2 Placenta and imprinted genes

Imprinted genes are important regulators of placental growth and function. The placenta regulates prenatal development and the fetal environment by controlling the transfer of nutrients and waste between the mother and the developing fetus and producing developmentally important growth factors and hormones (Sandovici, Hoelle, Angiolini, & Constância, 2012). The placenta has a capacity to adapt genetic as well as environmental factors through epigenetic modifications, thereby affecting intrauterine growth and the newborn's size at birth (Sandovici et al., 2012). It is known that SGA or LGA newborns, or newborns with low birth weight, are at higher risk for perinatal mortality and morbidity and to develop cardiovascular and metabolic diseases in later life (Gluckman, Hanson, Cooper, Thornburg, 2008; Sandovici et al., 2012). Referring to this and to DOHaD hypothesis (Barker, 2004) the proper function and regulation of the placenta is important. Several imprinted genes have been displayed to be expressed in the placenta. Some of these genes have roles in development and growth of the placenta and others in the control of nutrient supply (Peters, 2014). Disruption of these genes has been seen to result in fetal or placental overgrowth or growth restriction. For example, in mouse studies, the knockout of the imprinted gene in the placenta has seen to result in IUGR and restricted placental growth or placental overgrowth (Frank et al., 2002; Lefebvre et al., 1998; Takahashi, Kobayashi, & Kanayama, 2000). Since imprinted genes are regulated through DNA methylation, it is suggested that the placental DNA methylation status has a significant role in the fetal growth. DNA methylation alterations have been linked to the control and expression of placental imprinted genes with IUGR (Ishida & Moore, 2013), SGA and LGA infants and differences in birth weight (Koukoura, Sifakis, & Spandidos, 2012; Kappil et al., 2015; Turan et al., 2012).

1.4 *IGF2/H19* imprinted gene locus

This thesis focused on the *Insulin growth factor 2 (IGF2)/H19* locus, which is an imprinted gene locus located at chromosome 11p15.5 in humans. IGF2 is a growth factor especially

important during pregnancy. It is expressed from the paternal allele in most normal tissues, particularly in the placenta (Nordin, Bergman, Halje, Engström, & Ward, 2014). When binding to the insulin growth factor 1 receptor (IGF1R) on the cell surface, it activates signaling cascades that promote the growth of the placenta and the transfer of nutrients from a mother to fetus, consequently affecting the fetal growth and newborn's size at birth (Baker, Liu, Robertson, & Efstratiadis, 1993; Fowden, Sibley, Reik, & Constancia, 2006; Randhawa & Cohen, 2005). The *H19* gene is maternally expressed and encodes a 2.3 kb large intergenic non-coding RNA (lincRNA), which is suggested to function as a growth suppressor of the placenta during gestation (Keniry et al., 2012). *IGF2* and *H19* are clustered at an imprinted region, *H19* locating downstream of *IGF2* (Nordin et al., 2014). In most somatic cells, *IGF2* and the neighboring *H19* are reciprocally imprinted; *IGF2* is silenced in the maternal allele, whereas *H19* is silenced in the paternal allele. They share common regulatory mechanisms and are co-expressed in endoderm- and mesoderm-derived tissues during embryonic development (Nordin et al., 2014).

The *IGF2/H19* imprinted gene locus employs insulator model to maintain imprinting. The gene locus is regulated by the *H19* imprinting control region, ICR1, upstream of *H19* and two nearby enhancer elements (Figure 4) (Bell & Felsenfeld, 2000; Hark et al., 2000). In mouse studies, *H19* ICR1 and its methylation state are suggested to play a crucial role in the maintenance of *Igf2/H19* imprinting (Thorvaldsen, Duran, & Bartolomei, 1998). At the maternal allele, ICR1 is unmethylated, whereas in the paternal allele it is methylated (Bell & Felsenfeld, 2000; Hark et al., 2000). In humans, ICR1 consists of seven binding sites of zinc-finger protein CCCTC-binding factors (CTCFs) that organizes the three-dimensional structure of a chromatin (Phillips & Corces, 2009). Unmethylated ICR1 on the maternal allele allows CTCF binding, which consequently blocks downstream enhancers to act on the *IGF2* promoter, hence silencing its expression and enabling *H19* expression. On the contrary, in the paternal allele methylated ICR1 prevents CTCF binding and allows the enhancers to act on the *IGF2* promoter enabling its expression and silencing *H19* expression (Bell & Felsenfeld, 2000; Hark et al., 2000). Changes in DNA methylation at binding sites of CTCFs of ICRs is associated with differential CTCF binding (Wang et al., 2012). In addition to *H19* ICR1, the *IGF2/H19* gene locus is regulated by differentially methylated regions (DMRs): the *H19* promoter region (*H19* DMR) and *IGF2* DMR0, DMR1 and DMR2. DMRs are suggested to function in the interaction between *H19* ICR1, consequently, affecting the chromatin structure and tissue-specific imprinted gene expression (Muller, Heeson, & Reik, 2004). In mouse, *igf2* placenta-specific DMR0 is maternally methylated and DMR1 and DMR2 of *igf2* and the *H19* promoter (*H19* DMR) are paternally methylated. The *H19* promoter is known to

influence on *H19* transcription and *igf2* DMRs on *igf2* transcription (Constância et al., 2000; Eden et al., 2001; Muller et al., 2004).

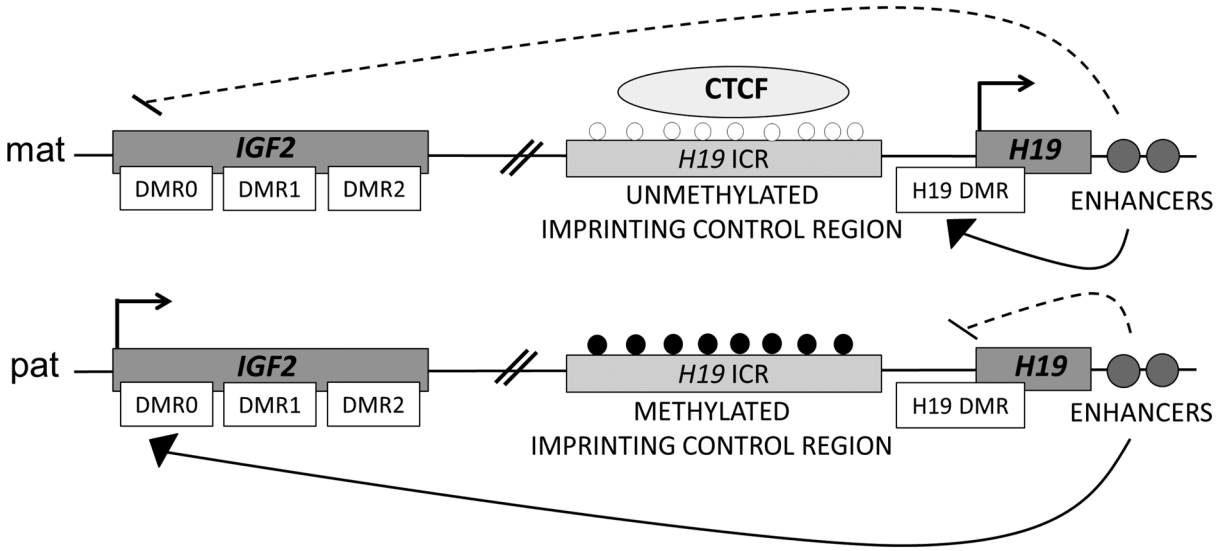


Figure 4. The regulation of the *IGF2/H19* gene locus. At *IGF2/H19*, ICR1 of the maternal allele is unmethylated, whereas in the paternal allele it is methylated. Unmethylation of ICR1 in the maternal allele allows zinc-finger protein CTCF binding, which consequently blocks downstream enhancers to act on the *IGF2* promoter, hence silencing its expression and enabling *H19* expression. The paternal allele acts vice versa. DMRs are suggested to function in the interaction between *H19* ICR1, consequently, affecting the chromatin structure and tissue-specific imprinted gene expression. Copy right obtained from Marjonen et al. (2017).

Since the expression of both, *IGF2* and *H19,* genes is controlled by *H19* ICR1, the adequate methylation of the region is important: altered DNA methylation at the *IGF2/H19* locus has been seen to cause abnormal fetal growth. Hypomethylation of *H19* ICR1 as well as the *H19* promoter and *IGF2* DMRs in the placenta has been associated with poor fetal growth and IUGR (Bourque, Avila, Peñaherrera, von Dadelszen, & Robinson, 2010; Guo et al., 2008; Koukoura et al., 2011). Moreover, hypomethylation of *H19* ICR1 is known to lead the overexpression of *H19*, downregulation of *IGF2,* and hence growth restriction and Silver–Russell syndrome (Gicquel et al., 2005) while hypermethylation has been associated with the overexpression of *IGF2* and downregulation of *H19* resulting in overgrowth and Beckwith-Wiedemann Syndrome (Soejima & Higashimito, 2013). In addition to methylation alterations, genotypic differences, such as rs4929984 and rs2071094 polymorphisms at the *IGF2/H19* locus, between individuals have been previously seen to associate with the birth size (Adkins et al., 2010; Petry et al., 2005, 2011). A single nucleotide polymorphism, rs10732516 G/A, which is in linkage to rs4929984 and rs2071094, has been seen to be associated with genotype-specific epigenetic profiles at the sixth binding sequence of CTCF (CTCF6) in both placental (Marjonen et al., 2017) and whole blood samples (Coolen et al., 2011; Rentería et

al., 2013). Marjonen et al. (2017) also found genotype-specific associations between the rs10732516 G/A polymorphism and alterations in the methylation levels at the CTCF6 of *H19* ICR1 in placentas as well as differences in the head circumference of alcohol-exposed newborns. In addition to *in utero* alcohol exposure, several environmental factors during early embryonic development, including maternal diet and tobacco smoking has been seen to influence on DNA methylation at the *IGF2/H19* locus (Loke et al., 2013).

1.5 Effects of ART on epigenome

In several studies, assisted reproductive technology has been associated with changes in epigenome and imprinted genes. It has been suggested that newborns conceived by IVF or IVF/ICSI have increased risk of having imprinting disorders, such as Angelman, Beckwith-Wiedemann, and Silver–Russell syndromes (Lazaraviciute et al., 2014; Ludwig et al., 2005; Maher et al., 2003). Interestingly, the Beckwith-Wiedemann cases associated with ART have been confined to epigenetic changes particularly at chromosome 11p15.5, such as in *IGF2/H19* imprinted gene locus, and the proportion of epigenetic changes among spontaneously conceived cases is much smaller (Weksberg, Shuman, & Smith, 2005).

The association between ART and imprinting disorders, and the important role of imprinted genes in the growth of the embryo, has led to a growing interest in the study between ART and epigenome. To date, several studies have been associated ART with DNA methylation and gene expression differences in imprinted genes. For example, in the genome-wide study, Katari et al. (2009) reported DNA methylation changes in the cord blood and placenta mostly in imprinted, but also in non-imprinted genes (Katari et al., 2009). Moreover, the effects of ART on epigenome are also supported by studies of *in vitro* culture conditions used in IVF treatment. In mouse studies, IVF and embryo culture conditions have been seen to affect methylation patterns and the gene expression of imprinted genes in mouse embryos (Doherty, Mann, Tremblay, Bartolomei, & Schultz, 2000; Fauque et al., 2007; Market-Velker, Fernandes, & Mann, 2010) and placentas (Fauque et al., 2010; Li et al., 2016).

Perhaps the most studied imprinted gene locus has been the *IGF2/H19* locus important for the growth and development of the embryo and placenta. Alterations in DNA methylation, especially at *H19* ICR1, have been identified: decreased DNA methylation levels at CTCF6 of *H19* ICR1 have been detected in placentas (Nelissen et al., 2013a), buccal epithelium (Loke et al., 2015) and cord blood mononuclear cells of newborns (Castillo-Fernandez et al.,

2017) conceived by IVF or IVF/ICSI. Moreover, differences in the gene expression of *IGF2* and *H19* in the placenta of IVF or IVF/ICSI newborns have been identified with variable results (Katagiri et al., 2010; Sakian et al., 2015; Turan et al., 2010). Furthermore, in addition to low birth weight in human studies (Dumoulin et al., 2010; Nelissen et al., 2012, 2013b), the embryo culture conditions used in IVF have also been seen to affect the methylation patterns of *H19* ICR1 and *H19* gene expression in mouse embryos (Doherty et al., 2000; Fauque et al., 2007; Market-Velker et al., 2010).

It has been challenging to distinguish whether the changes in epigenome are due to the ART procedures themselves, underlying infertility or both. In addition to ART newborns, methylation changes and differences in the expression of imprinted genes, such as in the *IGF2/H19* locus, have also been seen in newborns born to infertile couples (Litzky et al., 2017). Moreover, similar methylation alterations as in IVF or IVF/ICSI newborns, have been detected in sperm of infertile men, including hypomethylation at the CTCF6 of *H19* ICR1 (Boissonnas et al., 2010; Kobayashi et al., 2007; Marques et al., 2008). Furthermore, in the study by Castillo-Fernandez et al. (2017), DNA methylation changes were detected in several genes of IVF newborns that are previously linked to infertility, suggesting that the changes caused by IVF treatment may in part be reflected parental infertility. Some studies have also indicated an increased frequency of imprinting disorders in newborns born to infertile couples (Ludwig et al., 2005). However, the effects of ART on epigenome and imprinted genes have also been seen in mice (Li et al., 2016; Mann et al., 2004), which advocates that the IVF procedure, even without infertility, may induce epigenetic changes.

1.6 DNA methylation analyses

There are several methods and techniques developed for DNA methylation analysis over the past two decades. The technologies have been based on bisulfite conversion of DNA, selective restriction enzyme digestion of DNA, and affinity-based analyses that capture methylated DNA by antibodies or methyl binding domain proteins (Olkhov-Mitsel & Bapat, 2012). These approaches have been applied for example with PCR, sequencing, mass spectrometry and microarray platforms. The method should be chosen according to the research needs: the techniques differ with their robustness, sensitivity, specificity, high throughput capabilities and cost (Kurdyukov & Bullock, 2016). Others are more suitable for profiling a whole genome methylation status and others for detecting the methylation status of specific genes or differentially methylated regions.

Traditional bisulfite sequencing and Sequenom MassARRAY® EpiTYPER® assay based on sodium bisulfite conversion were used in this thesis for region-specific DNA methylation analyses. Sodium bisulfite conversion is based on the chemical treatment of single-stranded DNA with a sodium bisulfite under acidic condition, which deaminates the unmethylated cytosines into uracils leaving the methylated cytosines, 5-methylcytosines (5-MeCs), unchanged. The conversion and thereby the methylation can be detected from the genetic code by sequencing, for instance (Clark, Harrison, Paul, & Frommer, 1994; Clark, Statham, Stirzaker, Molloy, & Frommer, 2006). The sodium bisulfite conversion method is considered as simple, robust, specific and sensitive and can be applied in a variety of downstream methods (Ollikainen, 2011, p. 293). Advantages of bisulfite conversion-based methods include the possibility for a quantitative DNA methylation analysis, SNP genotyping as well as methylation detection in a single CpG resolution and identification of differentially methylated regions (Olkhov-Mitsel & Bapat, 2012).

1.6.1 Bisulfite sequencing

The traditional bisulfite sequencing was first introduced by Frommer et al. (1992) and optimized by Clark et al. (1994, 2006). The method is considered as the gold standard in DNA methylation studies since it enables reliably methylation detection in target sequence in a single CpG resolution. The information can be used to create single-stranded DNA methylation maps. The methylation profiles are also possible to detect in an allele-specific manner when the target sequence contains sequence variation or SNP, which is useful when studying the methylation of imprinted genes.

The bisulfite sequencing protocol (Frommer et al., 1992; Clark et al., 1994, 2006) is started by sodium bisulfite conversion that is followed by PCR amplification of the target sequence. Since the conversion of cytosines to uracils generates non-complementary strands, the PCR is done using primers, specific either the sense or antisense strands. The thymines, as well as the uracils, are amplified as thymines and the cytosines are amplified as cytosines. After the PCR, the products can be directly sequenced, which offers information about the average methylation profile of the target sequence in a population of cells, or the product can be cloned prior the sequencing, which provides the information about the methylation profile of the target sequence in a single cell. The DNA methylation data is analyzed using bioinformatic tools, such as BiQ Analyzer, that can be used for alignment as well as visualization and quality control of the DNA methylation data (Bock et al., 2005).

Even the bisulfite sequencing is considered as a gold standard, the protocol includes some considerable errors and challenges (Ollikainen, 2011, p. 295–299). Above all, the method is very labor-intensive and time-consuming, hence easily biased due to the low number of samples analyzed. Moreover, the errors in conversion, PCR and cloning may lead to inaccurate methylation data. In bisulfite conversion, poor-quality DNA or incomplete denaturation of DNA into single-stranded DNA may disrupt the conversion of unmethylated cytosines and result in a low conversion rate (Ollikainen, 2011, p. 295–299). Errors in PCR amplification may result in false methylation profiles and arise due to incorrect amplification of uracils, since the *Taq* DNA polymerase, used in amplification, does not have a proofreading property (Warnecke et al., 2002). Furthermore, the PCR amplification may prefer the unmethylated DNA strand over the methylated DNA strand, due to a higher C/G content, resulting in a PCR bias and inaccurate DNA methylation data (Warnecke et al., 1997). Thus, careful primer and PCR optimization are crucial. The errors in bisulfite sequencing may be also due to the cloning bias, in which the PCR product is preferentially cloned (Warnecke et al., 2002).

1.6.2 EpiTYPER

Sequenom MassARRAY® EpiTYPER®, developed by Ehrich et al. (2005), is a mass spectrometry-based bisulfite sequencing method for high-throughput and quantitative analysis of region-specific DNA methylation as well as for SNP genotyping. The method has good reproducibility, accuracy, and sensitivity allowing the detection of methylation difference as low as ~5%. The results of the methylation profiles are comparable to bisulfite sequencing (Coolen, Statham, Gardiner-Garden, & Clark, 2007). However, compared to bisulfite sequencing the method is less time-consuming and useful especially when the large number of samples or regions are needed to be analyzed.

The EpiTYPER protocol (Figure 5) is started by bisulfite conversion of DNA and followed by PCR amplification using reverse primer containing a T7-promoter tag, which is used for transcription of the reverse strand into a single-stranded RNA product. Bisulfite conversion changes unmethylated C to U, which is further changed to T in PCR amplification. On the contrary, methylated C remains C after conversion and PCR amplification. The RNA intermediate step transcripts T to A and C to G. Subsequently, the RNA product is cleaved base-specifically (C or T) with RNase A to generate fragments that differ with their lengths and masses. The cleaved fragments, containing one or more methylation sites, can be separated with their masses since the mass difference between A (originally unmethylated

CpG) and G (originally methylated CpG) is 16 Da. The mass differences are collected and analyzed by matrix-assisted laser desorption/ionization time-of-light (MALDI-TOF) mass spectrometry system and the data is analyzed with the EpiTYPER software. The average methylation levels of samples are given for CpG units that refers to a cleavage product containing one or multiple CpG sites depending on the cleavage pattern (Ollikainen, 2011, p. 305–309).

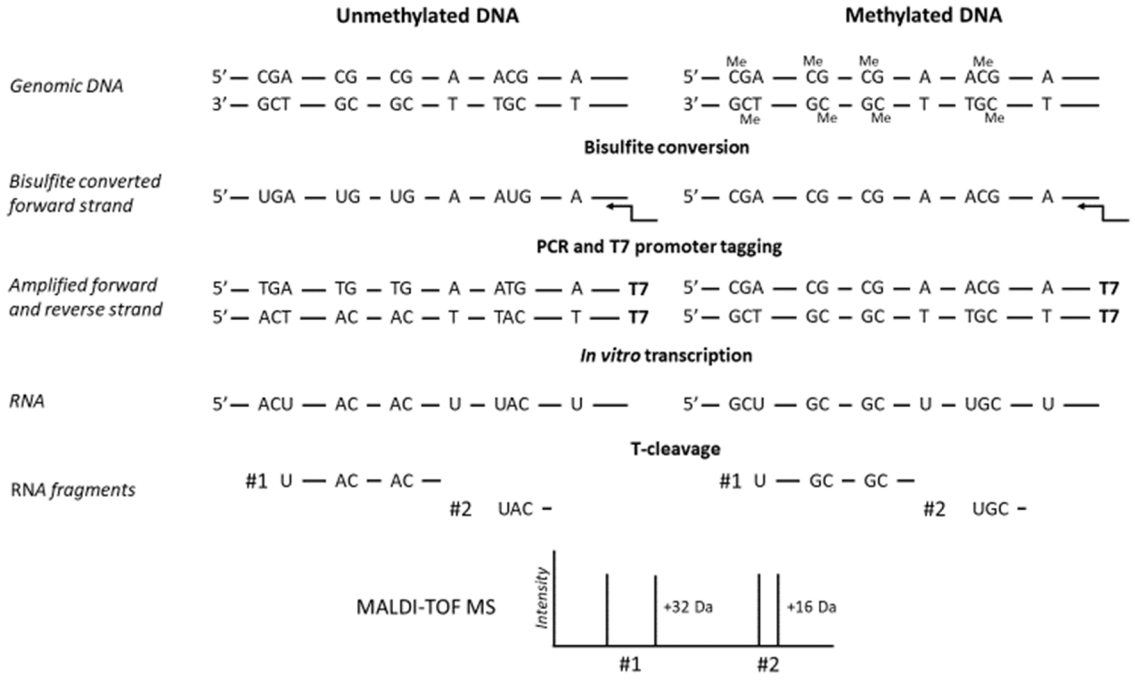


Figure 5. The overview of the MassARRAY® EpiTYPER® assay. The EpiTYPER protocol is started by bisulfite conversion of DNA and followed by PCR amplification and T7-promoter tagging. T7-promoter tagged reverse strand is transcripted into a single-stranded RNA product. The RNA product is cleaved base-specifically (T-cleavage in this example) to generate fragments that differ with their lengths and masses. The mass differences are collected and analyzed by MALDI-TOF mass spectrometry. The bisulfite conversion changes unmethylated C to U, which is further changed to T in PCR amplification. On the contrary, methylated C remains C after conversion and PCR amplification. The RNA intermediate step transcripts T to A and C to G. The mass difference between A (originally unmethylated CpG) and G (originally methylated CpG) is 16 Da. Modified from Ollikainen (2011, p. 306).

Even EpiTYPER is less labor-intensive than traditional bisulfite sequencing, it requires multiple working steps and specialized equipment. Moreover, the method cannot provide allele-specific information unlike the traditional bisulfite sequencing method (Ollikainen, 2011, p. 311–312). As in bisulfite sequencing, in EpiTYPER, the errors in conversion and PCR may result in inaccurate methylation data, which underlines the importance of the careful primer and PCR optimization (Ollikainen, 2011, p. 308).

2 Aims of the study

This thesis had two aims. The first aim was to study whether ART has effects on DNA methylation in the placenta and the second aim was to study whether ART has effects on the phenotype of newborns.

2.1 Effects of ART on DNA methylation in the placenta

Due to the possible alterations in epigenome of newborns conceived by ART, the aim was to study the effects of ART on DNA methylation in the placenta. To study these effects, aim was to analyze placental tissue samples of ART and spontaneously conceived newborns and explore total DNA methylation levels at the sixth binding sequence of CTCF (CTCF6) of *H19* ICR1 of the *IGF2/H19* imprinted gene locus, important for the growth and development of the embryo and placenta. Moreover, the aim was also to study total DNA methylation levels at *H19* promoter region (*H19* DMR) of *IGF2/H19* locus and, to observe the global methylation state in placenta, also at Long interspersed nuclear elements (LINE-1). The DNA methylation examination also aimed to study whether the possible alterations in DNA methylation in placentas of ART newborns associates with a rs10732516 G/A polymorphism locating at CTCF6 of *H19* ICR1. Thus, genotype-specific DNA methylation levels at *H19* ICR1, *H19* DMR and LINE-1 of ART and control placental samples were explored. Furthermore, allele-specific DNA methylation levels at *H19* ICR1 were explored in placental samples, but also in white blood cell (WBC) samples, to explore whether the possible changes in DNA methylation is similar in extra embryonic placental cells and embryonic blood cells of the newborns.

2.2 Effects of ART on the phenotype of newborns

Due to the decreased birth weight of IVF and IVF/ICSI newborns derived from fresh embryo transfer and due to increased birth weight of IVF and IVF/ICSI newborns derived from frozen embryo transfer (FET), the aim was to study the effects of ART on the phenotype of newborns. To study these effects, differences in birth weight, length and head circumstance of ART newborns were explored using international growth standards. Moreover, placental weights were examined. To reveal potential genotype-specific effects of ART, the aim was to explore the phenotype of newborns also according to the rs10732516 G/A polymorphism.

3 Materials and methods

3.1 Sample collection and study design

The study included placental and umbilical cord blood samples from Finnish and placental samples from Estonian newborns, which had been previously collected for this study. Finnish samples from ART pregnancies had been collected during years 2013–2017 (Marjonen et al., submitted) and Estonian samples from ART pregnancies during years 2016–2017. Couples had been applied to fertilization treatment in Fertility clinic of the Family Federation of Finland, Reproductive Medicine Unit of Helsinki University Central Hospital, Finland or Tartu University Hospital, Estonia. Fertilization had been done using *in vitro* fertilization (IVF) or *in vitro* fertilization with intracytoplasmic sperm injection (ICSI). Embryo transfer had been done using fresh embryos or using frozen embryos fertilized earlier (FET). Finnish (Marjonen et al., 2017) and Estonian control samples from spontaneous pregnancies had also been collected previously during years 2013–2015 in Helsinki University Central Hospital, Finland and Tartu University Hospital, Estonia. All the ART and control samples were from Finnish and Estonian newborns of Caucasian origin.

Altogether, the study included Finnish and Estonian placental tissue samples from 62 IVF and IVF/ICSI freshly transferred embryos and 24 IVF and IVF/ICSI frozen transferred embryos (FET) and 157 placental tissue samples derived from spontaneous pregnancies. Umbilical cord blood samples were also collected from 4 ART (1 IVF, 2 IVF/ICSI and 1 IVF/FET) derived pregnancies and 4 from spontaneous pregnancies of Finnish couples. For genotype-specific examination, samples were divided into four groups according to the rs10732516 G/A polymorphisms at CTCF6 of *H19* ICR1: G/G, paternal G/maternal A (patG/matA), paternal A/maternal G (patA/matG) and A/A. The summary of samples of the study and ART treatments used is shown in Table 1 and more detailed information about ART samples in Appendix A.

Table 1. The summary of samples of the study and ART treatments used.

ART treatment	Finnish newborns	Estonian newborns	Total
Fresh: IVF	23	21	44
Fresh: IVF/ICSI	6	12	18
FET: IVF	12	3	15
FET: IVF/ICSI	6	3	9
Total of ART samples	47	39	**86**
Control samples	100	57	**157**

3.2 Sample preparation

Genomic DNA of placental samples, stored in RNAlater® (Thermo Fisher Scientific) at -80 °C, from Finnish newborns were extracted using commercial QIAamp Fast DNA Tissue Kit (Qiagen) according to manufacturer's instructions with following exceptions. One to four pieces (three on average) of placental tissue samples (approximately 50, 25 or 12,5 mg of each) were weighed and pooled into Tissue Disruption Tubes supplied. The extraction was performed in two QIAamp Mini spin columns since one column maximum was 25 mg. For this reason, the lysis solution and Buffer MVL was used in double. After elution step, the eluate was combined from the two tubes and the DNA concentration was determined using NanoDrop Lite Spectrophotometer (Thermo Scientific). The extraction of placental samples of Estonian newborns as well as the WBC and genomic DNA extraction from umbilical cord blood samples had been done earlier.

3.3 Genotype analysis

To divide the samples into four groups (G/G, patG/matA, patA/matG and A/A) according to the rs10732516 G/A polymorphisms, and discern the heterozygotes from homozygotes, the placental samples were genotyped by Sanger sequencing. According to previous sequencing analyses, the paternal and maternal alleles amplify unevenly, which enabled the distinction between the heterozygous genotypes: the peak of rs10732516 A in sequence of patA/matG genotype was lower compared to patG/matA genotype. Altogether, all the 86 ART placental samples (47 from Finnish and 39 from Estonian newborns) and 57 control placental samples (from Estonian newborns) were genotyped. The 100 Finnish control placental samples had been genotyped previously by Marjonen et al. (2017).

For sequencing, placental DNA was amplified using PCR (HotStar PCR kit, Qiagen) according to manufacturer's instructions. From 100 to 300 ng of template DNA was used in one PCR reaction for each sample. Primers obtained from previous publication (Marjonen et al., 2017) were used as 0.5 µM in each 20 µl reaction. The PCR protocol and primers used are presented in Appendix B. PCR products, water control and DNA marker (ΦX174 DNA/BsuRI (HaeIII) marker, 9) were run in agarose gel electrophoresis to confirm the proper amplification. SAP-treatment (FastAP Thermosensitive Alkaline Phosphatase (1 U/µL), Thermo Scientific) was used for the purification of the PCR products according to manufacturer's instructions. For sequencing, 1.6 µl of 5 µM reverse primer was added into 5 µl of each purified PCR product and the sequencing was performed by Institute for Molecular Medicine Finland (FIMM).

3.4 Methylation analysis

3.4.1 EpiTYPER

To explore the total and genotype-specific DNA methylation levels at CTCF6 of *H19* ICR1, *H19* DMR and LINE-1 regions the methylation profiles of rs10732516 homozygous (A/A and G/G) and heterozygous (patG/matA and patA/matG) ART and control placental samples were analyzed by MassARRAY EpiTYPER (SEQUENOM Inc.). Altogether, the EpiTYPER analysis was performed for 62 ART derived (42 IVF: 24 Finnish (5 FET) and 17 Estonian (3 FET) and 20 IVF/ICSI: 6 Finnish (3 FET) and 15 Estonian (3 FET) newborns) and 60 control (40 from Finnish and 20 from Estonian newborns) placental samples.

Bisulfite conversion was performed using EZ-96 DNA Methylation™ kit (Zymo Research) according to manufacturer's instructions with 1000 ng of genomic DNA of each sample. The conversion was followed by PCR (HotStar PCR kit, Qiagen) according to manufacturer's instructions and performed in three independent reactions to minimize the possible PCR bias. Primers for the target sequences were obtained from previous publications (Ollikainen et al., 2010; Wang et al., 2010) and used as 0.4 µM in each 10 µl reaction. The PCR protocol and the primers used are presented in Appendix B. To confirm the proper amplification, six PCR reactions (three triplicates of two samples) of each target sequence, water control and DNA marker (ΦX174 DNA/BsuRI (HaeIII) marker, 9) were run in agarose gel electrophoresis. The three PCR reactions were pooled and the further procedures and the EpiTYPER analysis were performed by FIMM. With EpiTYPER analysis, it was also possible to confirm

the genotypes of the samples using genotypic methylation levels detected in unit CpG10 of *H19* ICR1. In patG/matA genotype the methylation level was ~0.80, in G/G ~0.30, in patA/matG ~0.02 and in A/A there was no value.

3.4.2 Bisulfite sequencing

To explore the genotype- and allele-specific DNA methylation levels, as well as to confirm the heterozygous genotypes, CTCF6 of *H19* ICR1 of 14 heterozygous ART placental samples (6 with patG/matA genotype: 4 IVF, 1 IVF/FET, 1 IVF/ICSI/FET and 8 with patA/matG genotype: 4 IVF, 3 IVF/ICSI, 1 IVF/FET) were bisulfite sequenced. Bisulfite sequencing for 12 control placental samples (Marjonen et al., 2017) (6 from patG/matA and 6 from patA/matG) and for 8 WBC samples (1 IVF, 2 IVF/ICSI, 1 IVF/FET and 4 controls (all from patA/matG genotype)) had been done earlier. All the samples subjected to bisulfite sequencing were from Finnish newborns.

Bisulfite conversion was performed using commercial EZ DNA Methylation™ kit (Zymo Research) according to manufacturer's instructions with 500 ng of genomic DNA. Two separate conversions were performed and pooled afterwards. After conversion, PCR (HotStar PCR kit, Qiagen) was performed according to manufacturer's instructions for each pooled sample in three independent reactions to minimize the possible PCR bias. Primers, obtained from a previous publication (Coolen et al., 2007), allowed the potential polymorphisms detected in sites CpG17,18,19,20 and were used as 0.5 µM in each 20 µl PCR reaction. The PCR protocol and primers used are presented in Appendix B. PCR products, water control and DNA marker (ΦX174 DNA/BsuRI (HaeIII) marker, 9) were run in agarose gel electrophoresis to confirm the proper amplification. The three PCR reactions of each sample were pooled and purified from the agarose gel using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to manufacturer's instructions.

The purified PCR fragments were cloned using pGEM®-T Easy Vector system (Promega) in *E. Coli*. The ligation and transformation protocol used is presented in Appendix C. The cells carrying the vector were cultured on ampicillin-containing Lysogeny broth selection plates (two or four per each individual) and the colonies containing the proper recombinant-DNA-clones were screened using alfa-complementation. Pure cultures (in Lysogeny broth + 1 mg/ml ampicillin) were made from 50–80 colonies from each individual. The plasmids containing the recombinant-DNA were purified using NucleoSpin® Plasmid EasyPure kit (MacheMacherey-Nagel) according to manufacturer's instructions. For sequencing, 1.6 µl of

5 µM pGEM-T® reverse primer (Promega) was added into 5 µl of the purified plasmids and the sequencing was performed by FIMM. Methylation profiles of the sequences were analyzed by BIQ Analyzer (Bock et al., 2005) and the sequences having conversion rate lower than 90% were not considered. Due to the heterozygosity and differentially methylated CTCF6 of *H19* ICR1 as the result of imprinting, the paternal and maternal alleles were able to differentiate from each other.

3.5 Phenotype analysis

The effects of ART on the phenotype of the Finnish and Estonian newborns were explored by examining birth weight (g), length (cm) and head circumference (cm) using international growth standards (Fenton Preterm Growth Chart by PediTools (http://peditools.org/)). The growth chard considers the gestational age at birth and gender when calculating the SD (z-score) of birth measures (Fenton et al., 2013). Measures deviating more than ±2 SDs are considered abnormal: newborns small for gestational age (SGA) have -2 SDs below the mean birth weight for gestational age and by contrast newborns large for gestational age (LGA) have +2 SDs over the mean birth weight for gestational age (Clayton et al., 2007).

3.6 Statistical analysis

All the statistical analyses were performed using SPSS software for Windows version 22.0 or GraphPad Prism 7 software (GraphPad Software, Inc.). When the samples were divided into the four genotypes, a *chi*-square test was used to test the frequency of G and A alleles of the rs1072516 polymorphism between ART and control samples. In methylation analysis, the non-parametric Mann–Whitney U test was used to compare the methylation levels of CpG sites analyzed by bisulfite sequencing and Student's t-test to compare the methylation levels of CpG units analyzed by EpiTYPER. The methylation data is presented as the mean with ± SD for a normal distribution of variables. Nominal *P*-values were considered significant when <0.05 and Bonferroni correction was used for multiple testing corrections. Student's t-test or Two-way and One-way ANOVA with Bonferroni *post hoc* for multiple testing correction were used to compare the birth measures between the groups and to test the interaction effect between the Finnish and Estonian study populations.

4 Results

4.1 Total DNA methylation levels at *H19* ICR1, *H19* DMR and LINE-1

The effects of ART on total DNA methylation levels at CTCF6 of *H19* ICR1 of placental samples were examined by MassARRAY EpiTYPER (Appendix D). No significant differences were observed between ART and control samples. By using the EpiTYPER method, the total methylation levels were also examined at the *H19* promoter (*H19* DMR) and, to observe the global methylation state in placenta, also at Long interspersed nuclear element (LINE-1) (Appendix D). No significant differences were observed in the total methylation levels in these regions between ART and control samples either.

4.2 Genotype-specific DNA methylation levels at *H19* ICR1 and *H19* DMR

Next, to study whether the effects of ART on DNA methylation are genotype-specific, samples were divided into four groups according to the rs10732516 G/A polymorphisms at CTCF6 of *H19* ICR1: G/G, patG/matA, patA/matG and A/A. The Frequency of the G and A alleles of the polymorphism is almost even in the Finnish population (G=0.47 and A=0.53) (The 1000 Genomes Project Consortium, 2015) as well as between ART and control samples of this study ($X^2(3)=5.52$, $P=0.138$, *chi*-square test).

4.2.1 EpiTYPER

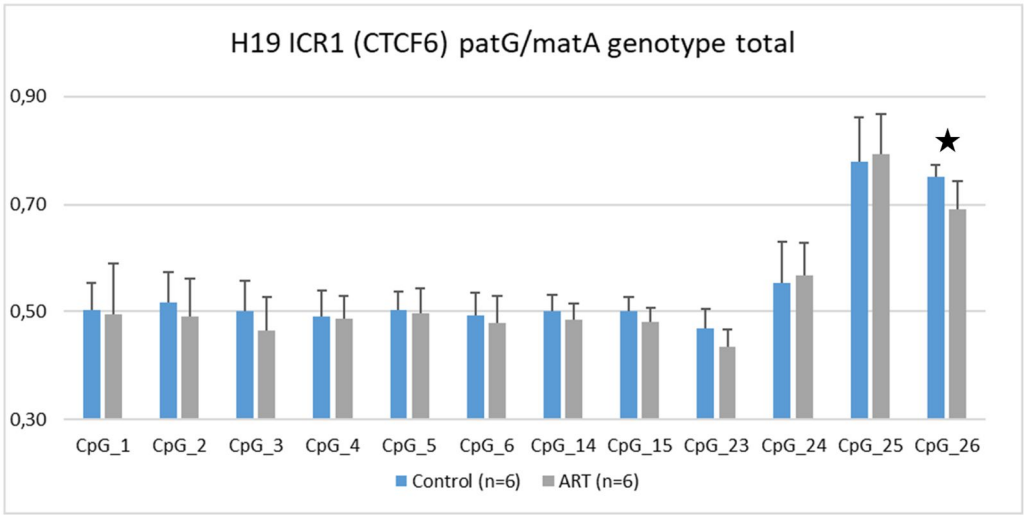
The genotype-specific DNA methylation levels at CTCF6 of *H19* ICR1 in placental samples were first examined by EpiTYPER method (Appendix D). By this method, the genotype-specific methylation levels were examined within all the four genotypes. However, no changes between ART derived and control placentas were detected. By using the EpiTYPER method, the genotype-specific methylation levels were also examined at *H19* DMR. According to the results, increased methylation levels in ART derived placentas were detected in units CpG3 and CpG6 in A/A genotype (nominal *P*-values=0.03 and 0.05, respectively, Student's t-test). However, the results were not significant after Bonferroni multiple testing corrections.

4.2.2 Bisulfite sequencing

Next, the genotype-specific DNA methylation levels were examined by bisulfite sequencing (Appendix E). By this method, the methylation levels were only examined within the two heterozygous genotypes, patG/matA and patA/matG, to be able to distinguish paternal and maternal alleles. In bisulfite sequencing, apparent PCR bias was detected. The amplification of hypomethylated maternal allele was favorited over hypermethylated paternal allele especially in rs10732516 patA/matG genotype. Therefore, the average methylation percentages were calculated separately for both alleles and then the total methylation levels were calculated for each CpG site (CpG1-27) weighing both alleles equally.

According to the results, decreased DNA methylation levels at six CpG sites, CpG1, CpG2, CpG3, CpG5, CpG14 and CpG24, at CTCF6 in ART derived placentas in patA/matG genotype were observed (nominal *P*-values=0.008, 0.02, 0.001, 0.013, 0.013, 0.029, respectively, Mann-Whitney U) (Figure 6B). By contrast, in patG/matA genotype, increased methylation level at site CpG26 was detected (nominal *P*-value=0.041, Mann-Whitney U) (Figure 6A). The results were not significant after Bonferroni multiple testing corrections, however, the decreased methylation levels in patA/matG were constant in several CpG sites (Figure 6B).

A



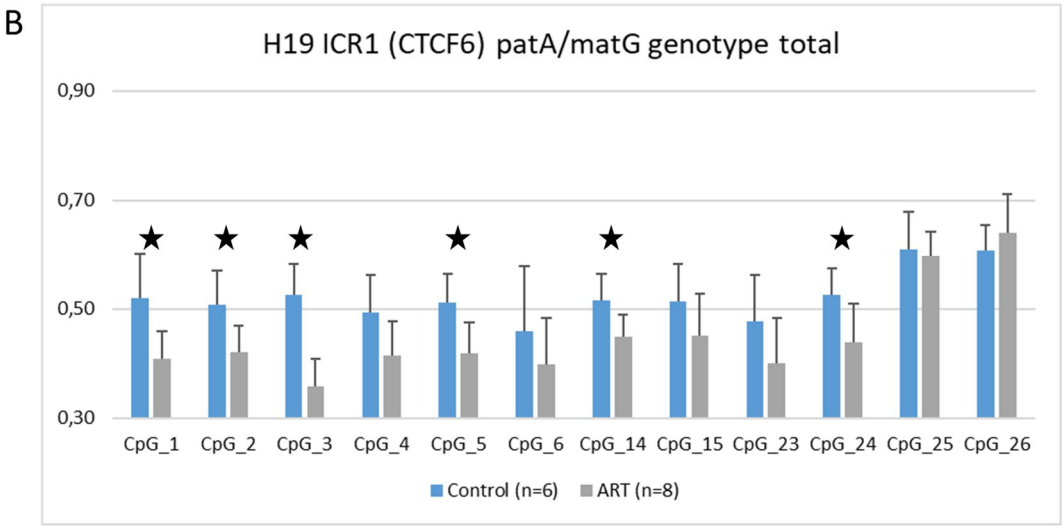
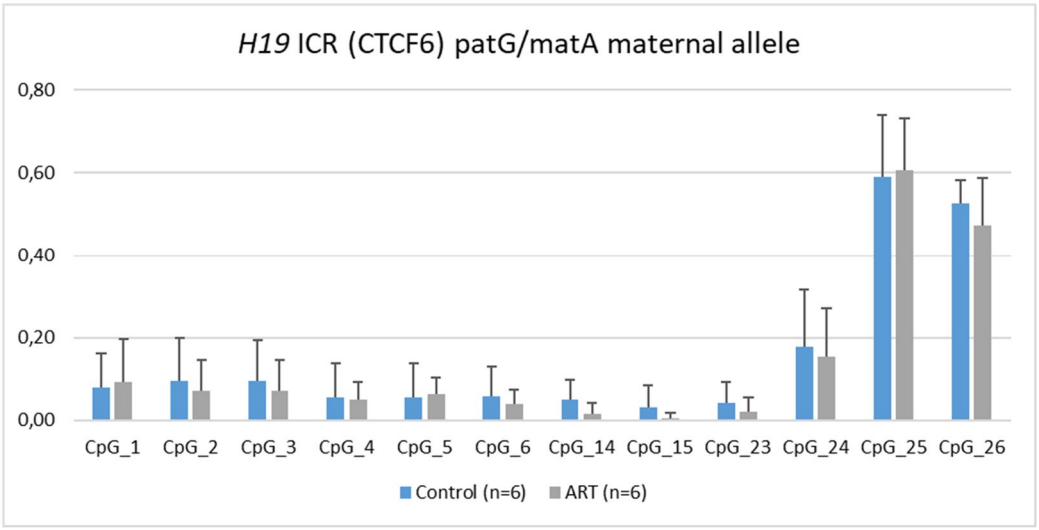


Figure 6. Genotype-specific DNA methylation levels of 12 CpG sites at CTCF6 of *H19* ICR1 of control and ART derived placentas analyzed by bisulfite sequencing. A. Methylation levels of patG/matA genotype in ART derived placentas (6) compared to control placentas (6). Star at CpG26 represents increased methylation level in ART derived placentas (nominal *P*-value=0.041, Mann-Whitney U) **B.** Methylation levels of patA/matG genotype in ART derived placentas (8) compared to controls (6). Stars at sites CpG1, CpG2, CpG3, CpG5, CpG14 and CpG24 represent decreased methylation levels in ART derived placentas (nominal *P*-values=0.008, 0.02, 0.001, 0.013, 0.013, 0.029, respectively, Mann-Whitney U). Modified from Marjonen et al., submitted.

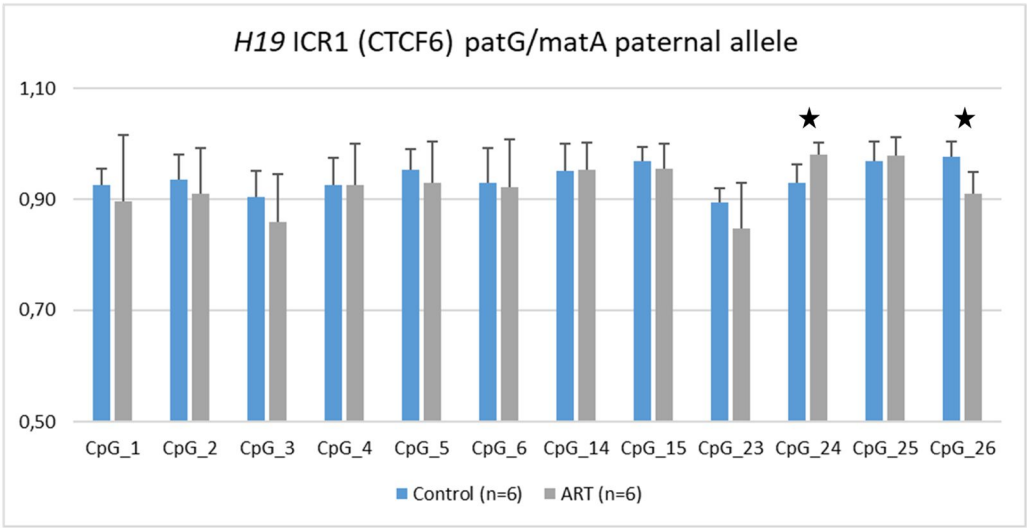
4.3 Allele-specific DNA methylation levels at *H19* ICR1

Finally, allele-specific effects of ART on DNA methylation at CTCF6 of *H19* ICR1 were examined by bisulfite sequencing (Appendix E). The DNA methylation levels were only examined within the patG/matA and patA/matG genotypes. First, the methylation levels were examined in placental samples. Decreased methylation levels in paternal allele of patA/matG genotype in ART derived placentas were detected at sites CpG1, CpG2, CpG3, CpG4 CpG5, CpG14, CpG24 and CpG25 (nominal *P*-values=0.013, 0.013, 0.001, 0.029, 0.013, 0.003, 0.005, 0.029, respectively, Mann-Whitney U) (Figure 7D). By contrast, in maternal allele of patA/matG genotype, increased methylation level was detected at site CpG26 (nominal *P*-value=0.005, Mann Whitney U) (Figure 7C). Moreover, in the paternal allele of patG/matA genotype, minor changes were detected in ART derived placentas: increased methylation level at site CpG24 and decreased methylation level at site CpG26 (nominal *P*-values=0.026 and 0.015, respectively, Mann-Whitney U) (Figure 7B). No changes were detected in the maternal allele of patG/matA genotype (Figure 7A). However, again the results were not significant after Bonferroni multiple testing corrections, but the methylation levels in the paternal allele of patA/matG genotype were consistently decreased in several CpG sites (Figure 7D).

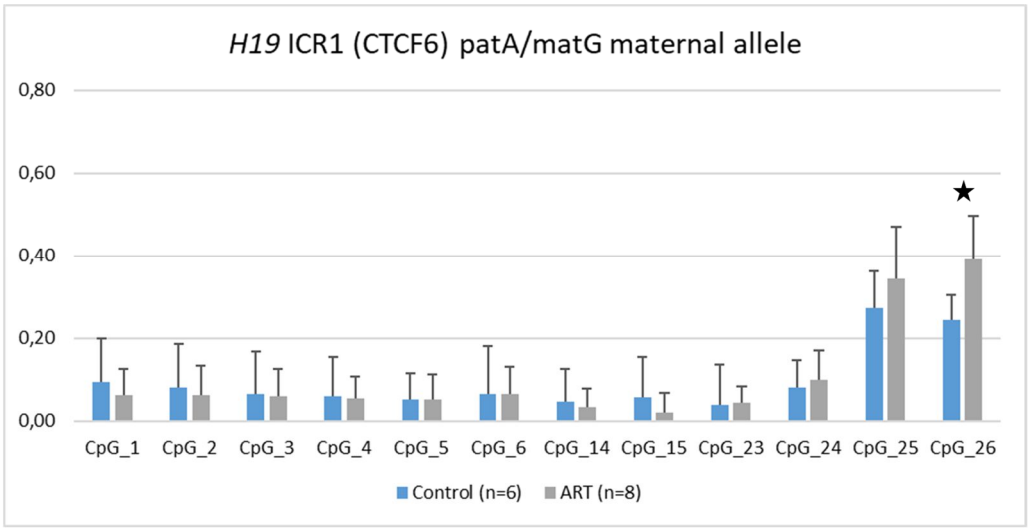
A


H19 ICR (CTCF6) patG/matA maternal allele
0,80
0,60
0,40
0,20
0,00
CpG_1 CpG_2 CpG_3 CpG_4 CpG_5 CpG_6 CpG_14 CpG_15 CpG_23 CpG_24 CpG_25 CpG_26
Control (n=6)
ART (n=6)

B


H19 ICR1 (CTCF6) patG/matA paternal allele
1,10
0,90
0,70
0,50
★
★
CpG_1 CpG_2 CpG_3 CpG_4 CpG_5 CpG_6 CpG_14 CpG_15 CpG_23 CpG_24 CpG_25 CpG_26
Control (n=6)
ART (n=6)

C


H19 ICR1 (CTCF6) patA/matG maternal allele
0,80
0,60
0,40
0,20
0,00
★
CpG_1 CpG_2 CpG_3 CpG_4 CpG_5 CpG_6 CpG_14 CpG_15 CpG_23 CpG_24 CpG_25 CpG_26
Control (n=6)
ART (n=8)

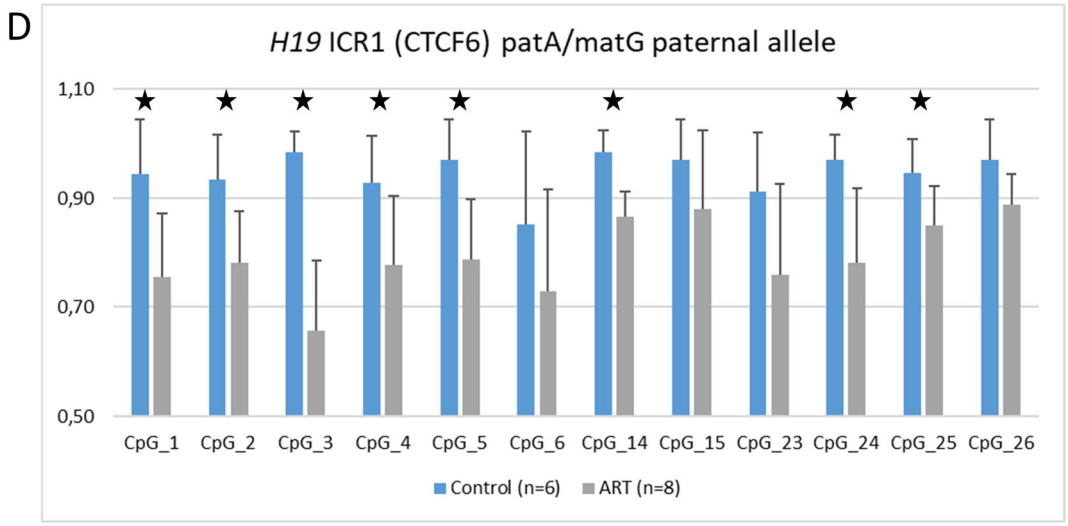


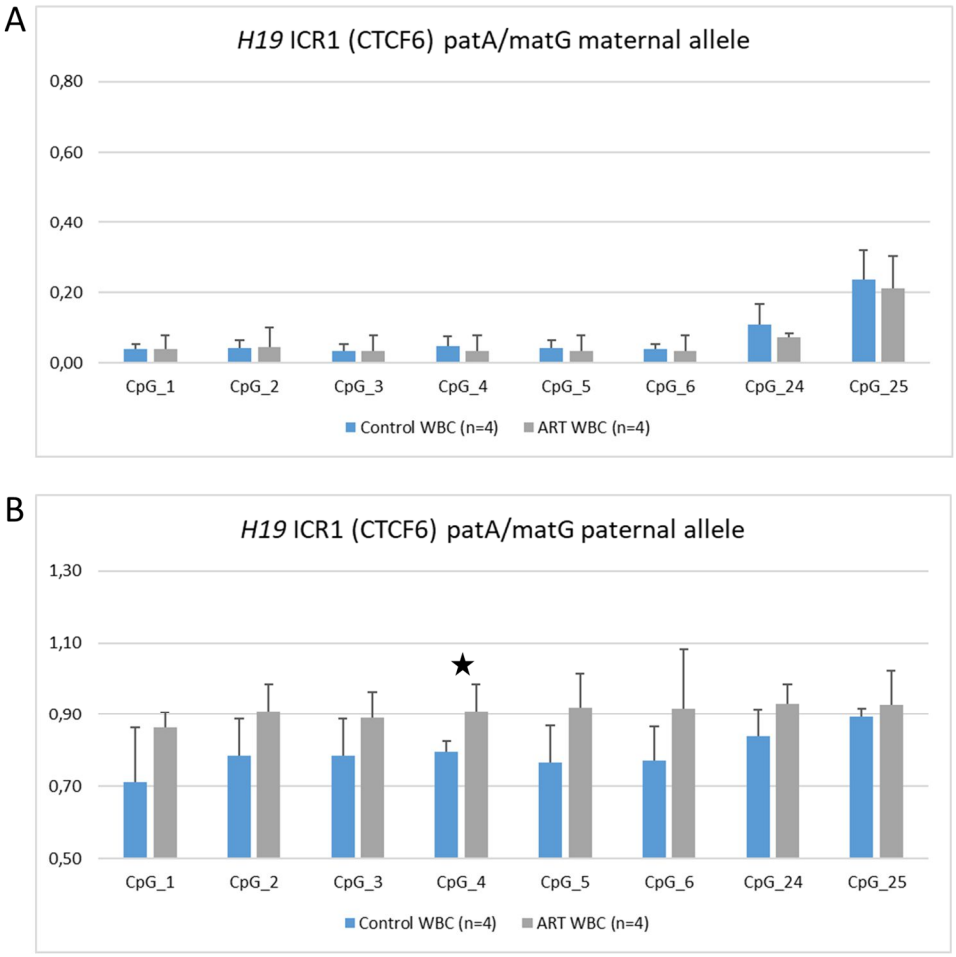
Figure 7. Allele-specific DNA methylation levels of 12 CpG sites at CTCF6 of *H19* ICR1 of control and ART derived placentas analyzed by bisulfite sequencing. A. Methylation levels in the maternal allele of patG/matA genotype in ART derived placentas (6) compared to control placentas (6). No differences were detected. **B.** Methylation levels in the paternal allele of patG/matA genotype in ART derived placentas (6) compared to controls (6). Star at site CpG24 represents increased methylation level in ART derived placentas (nominal *P*-value=0.026, Mann-Whitney U) and star at site CpG26 decreased methylation level in ART derived placentas (nominal *P*-value=0.015, Mann-Whitney U). **C.** Methylation levels in the maternal allele of patA/matG genotype in ART derived placentas (8) compared to controls (6). Star at site CpG26 represents increased methylation level in ART derived placentas (nominal *P*-value=0.005, Mann-Whitney U). **D.** Methylation levels in the paternal allele of patA/matG genotype in ART derived placentas (8) compared to controls (6). Stars at sites CpG1, CpG2, CpG3, CpG4 CpG5, CpG14, CpG24 and CpG25 represent decreased methylation levels in ART derived placentas (nominal *P*-values=0.013, 0.013, 0.001, 0.029, 0.013, 0.003, 0.005, 0.029, respectively, Mann-Whitney U). Modified from Marjonen et al., submitted.

Allele-specific methylation levels were also compared between the genotypes within control and ART placental samples. In control placentas, patA/matG genotype showed higher methylation levels compared to patG/matA genotype at sites CpG3 and CpG20 in paternal allele (nominal *P*-values=0.02 and 0.02, respectively, Mann Whitney U) and lower methylation levels at sites CpG25 and CpG26 in maternal allele (nominal *P*-values=0.004 and 0.002, respectively Mann Whitney U). In ART derived placentas, the paternal allele of patA/matG showed lower methylation levels compared to patG/matA genotype at several CpG sites: CpG2-5, CpG14, CpG24 and CpG25 (nominal *P*-values=0.02, 0.008, 0.03, 0.03, 0.008, 0.001, 0.001, respectively, Mann-Whitney U). Also, in the maternal allele, lower methylation level in placentas of patA/matG genotype compared to patG/matA genotype was observed at site CpG25 (nominal *P*-value=0.008, Mann-Whitney U). However, the results were not significant after Bonferroni multiple testing corrections.

The methylation profiles of ART derived and control placentas, examined by bisulfite sequencing, were also visualized by DNA methylation maps of 27 CpG sites at CTCF6 of *H19* ICR1 (Appendix F). The methylation maps illustrated the slightly hypomethylated

paternal allele of ART derived placentas compared to controls in patA/matG genotype. Furthermore, the methylation maps illustrated more efficiently PCR amplified maternal allele compared to the paternal allele of patA/matG genotype as also observed in previous publications (Coolen et al., 2011; Marjonen et al., 2017).

To examine whether the allele-specific effects of ART on DNA methylation at CTCF6 of *H19* ICR1 in the paternal allele of patA/matG genotype in placental samples can also be seen in embryonic cells, methylation levels of WBCs in the umbilical cord blood of newborns with patA/matG genotype were examined (Appendix E). By contrast to placental samples, slightly, but consistently increased methylation levels were observed in ART WBCs compared to controls. Specifically, the methylation level at site CpG4 in paternal allele was increased in ART WBCs (nominal *P*-value=0.03, Mann-Whitney U), however, not significantly after Bonferroni multiple testing correction (Figure 8B). No differences were detected in maternal allele (Figure 8A) or when the paternal and maternal alleles were calculated together (Figure 8C).



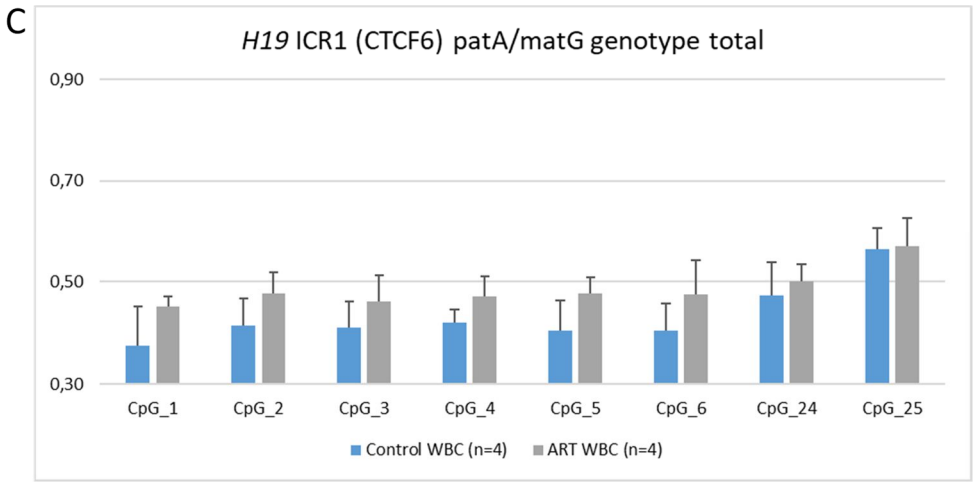


Figure 8. Allele-specific DNA methylation levels of 8 CpG sites at CTCF6 of *H19* ICR1 of ART-derived and control WBCs analyzed by bisulfite sequencing. A. Methylation levels in the maternal allele of patG/matA genotype in ART WBCs (4) compared to control WBCs (4). No differences were detected. **B.** Methylation levels in the paternal allele of patG/matA genotype in ART-derived WBCs (4) compared to control WBCs (4). Star at site CpG4 represents increased methylation level in ART-derived WBCs (nominal *P*-value=0.03, Mann-Whitney U). **C.** Methylation levels in ART WBCs (4) compared to control WBCs (4) when the paternal and maternal alleles in patA/matG genotype were calculated together. No differences were detected. Modified from Marjonen et al. submitted.

4.4 Phenotypes of newborns

4.4.1 Participants' characteristics

Significant differences were observed between ART and control groups in maternal age (*P*<0.001, Two-way ANOVA), maternal parity (*P*=0.001, Two-way ANOVA) and gestational age (*P*<0.001, Two-way ANOVA), but not in maternal BMI or in five minutes Apgar score. Also, there were no differences in gender between ART and control newborns. The study populations of Finnish and Estonian newborns differed significantly in maternal age (*P*<0.001, Two-way ANOVA), maternal parity (*P*=0.01, Two-way ANOVA) and in gestational age (*P*=0.002, Two-way ANOVA). However, the interaction effects were not significant (*P*=0.9, 0.8, 0.4, respectively, Two-way ANOVA). The study populations did not differ in maternal BMI or in five minutes Apgar score.

4.4.2 Effects of ART on the phenotype of newborns

To study the effects of ART on the phenotype of newborns, the birth weight, length and head circumference of ART derived, and control newborns were examined using international growth standards (Fenton et al., 2013). Moreover, the placental weights of the newborns

were compared. The birth measures were examined in two study groups: 1. controls and ART derived newborns and 2. controls, fresh (IVF and IVF/ICSI) and frozen embryo transfer (FET) (IVF and IVF/ICSI) derived newborns. In the study group of controls and ART derived newborns, there were significant differences in birth measures between the Finnish and Estonian study populations. The study populations differed in birth weight (P=0.007, Two-way ANOVA), birth length (P<0.001, Two-way ANOVA), head circumference (P=0.006, Two-way ANOVA) and placental weight. However, the interaction effects were not significant (P=0.8, 0.9, 0.1, 0.7, respectively, Two-way ANOVA) and the data was combined. There were also significant differences in the birth measures between the Finnish and Estonian study populations in the study group of controls, fresh embryo transfer and FET derived newborns. The study populations differed in birth length (P=0.001, Two-way ANOVA) and placental weight (P<0.001, Two-way ANOVA). However, the interaction effects were not significant (P=0.9 and 0.4. respectively, Two-way ANOVA) and the data was combined.

According to the results, any of the standard deviations of the birth measures, or the placental weight, did not differ significantly between ART and control newborns. However, when the ART group was divided into fresh embryo transfer and FET derived newborns, the placental weight differed significantly between the groups (P=0.04, Two-way ANOVA) (Figure 9D). Placentas of fresh derived newborns were smaller than placentas of controls (P=0.01, Bonferroni *post hoc*) and FET derived newborns (P<0.001, Bonferroni *post hoc*). Moreover, placentas of FET derived newborns were heavier than placentas of control newborns (P=0.01, Bonferroni *post hoc*). The standard deviations of other birth measures did not differ significantly between the groups (Figure 9A-9C).

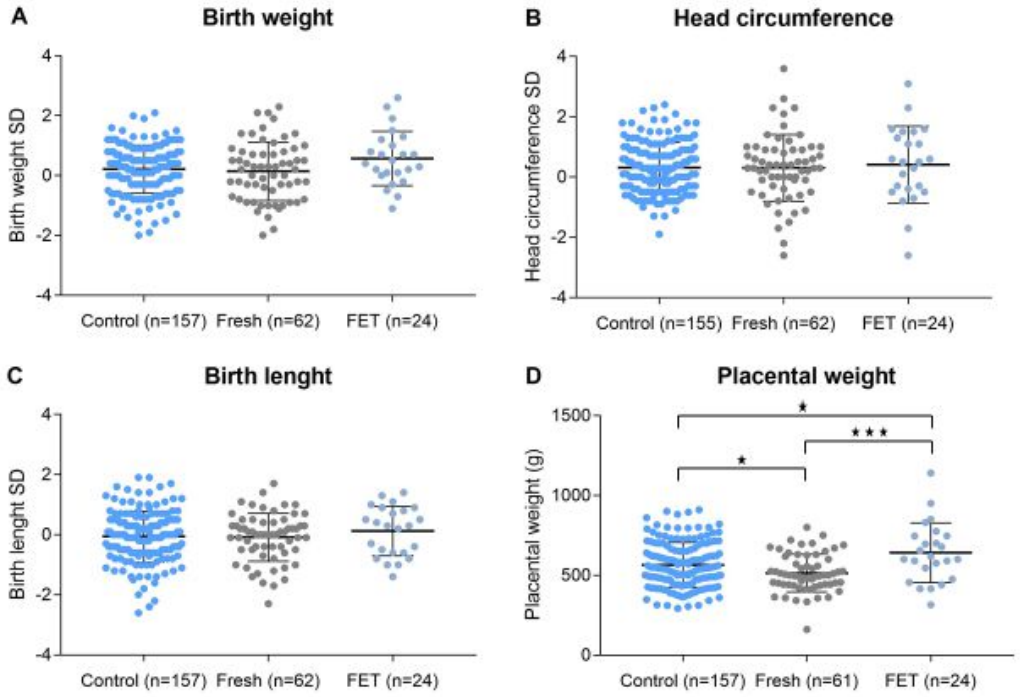


Figure 9. Birth measures of controls and fresh and frozen embryo transfer (FET) derived newborns. A. Birth weight SD **B.** Head circumference SD **C.** Birth length SD **D.** Placental weight (g). The placental weight differed significantly between the groups (*P*=0.04, Two-way ANOVA). Placentas of fresh embryo transfer derived newborns were smaller than placentas of controls (*P*=0.01, Bonferroni *post hoc*) and FET derived newborns (*P*<0.001, Bonferroni *post hoc*) and placentas of FET derived newborns were heavier than placentas of control newborns (*P*=0.01, Bonferroni *post hoc*). Stars represent: *P < 0.05, **P < 0.01, ***P ≤ 0.001 (Two-way ANOVA followed by Bonferroni *post hoc*).

4.4.3 Genotype-specific effects of ART on the phenotype of newborns

To study the genotype-specific effects of ART on the phenotype of newborns, international growth standards were again used to examine the birth weight, length and head circumference of newborns (Fenton et al., 2013). Furthermore, placental weights of the newborns were compared. First, the genotype-specific effects were compared between ART derived and control newborns. According to the results, the head circumference (SD) of ART derived newborns was smaller compared to controls with A/A genotype (*P*=0.04, Student's t-test) (Figure 10D), but not in other genotypes (Figure 10A-10C). Any other birth measures did not differ significantly between the groups.

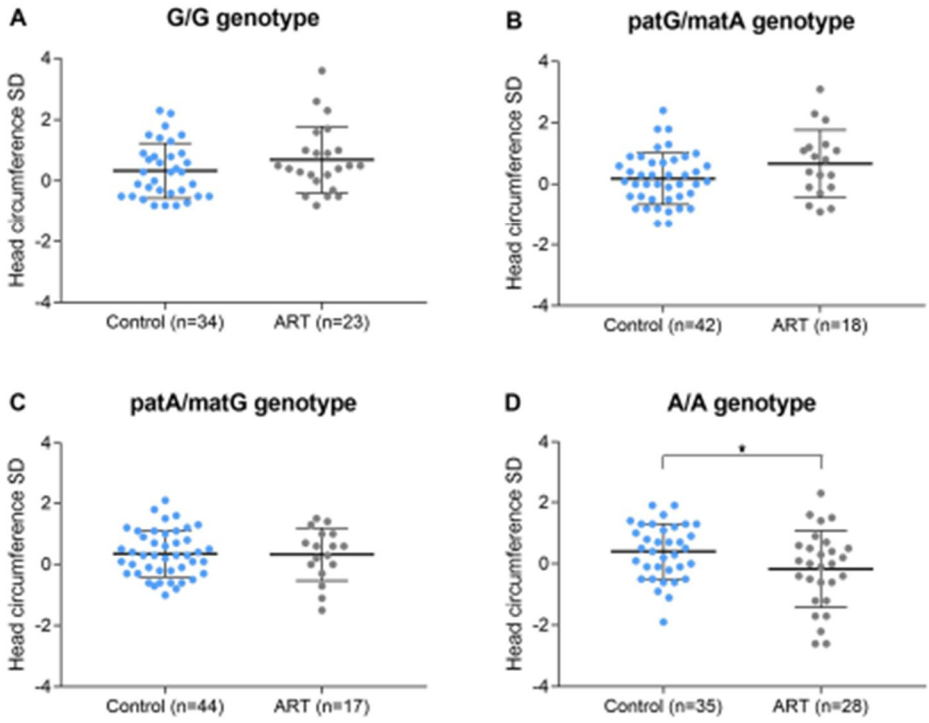


Figure 10. Genotype-specific effects in head circumference (SD) of control and ART derived newborns. A. G/G genotype. **B.** patG/matA genotype. **C.** patA/matG genotype. **D.** A/A genotype. The head circumference (SD) of ART derived newborns with A/A genotype was smaller compared to controls (*P*=0.04, Student's t-test). Stars represent: *P < 0.05, **P < 0.01, ***P ≤ 0.001 (Student's t-test).

Next, the ART group was divided again for further genotype-specific examination. Due to the low number of heterozygous (patG/matA, patA/matG) FET derived samples, the examination was done only with control, fresh embryo transfer and FET derived newborns with homozygous G/G and A/A genotypes. According to the results, birth weights (SD) and placental weights differed significantly between the groups in A/A genotype (*P*=0.02, *P*=0.006, respectively, One-way ANOVA). The birth weight (Figure 11B) and placental weight (Figure 11D) of FET derived newborns were higher compared to fresh derived newborns with A/A genotype (*P*=0.02, *P*=0.004, respectively, Bonferroni *post hoc*). The same difference was not seen in G/G genotype (Figure 11A and 11C). Significant differences in head circumference or in birth length were not detected between the groups.

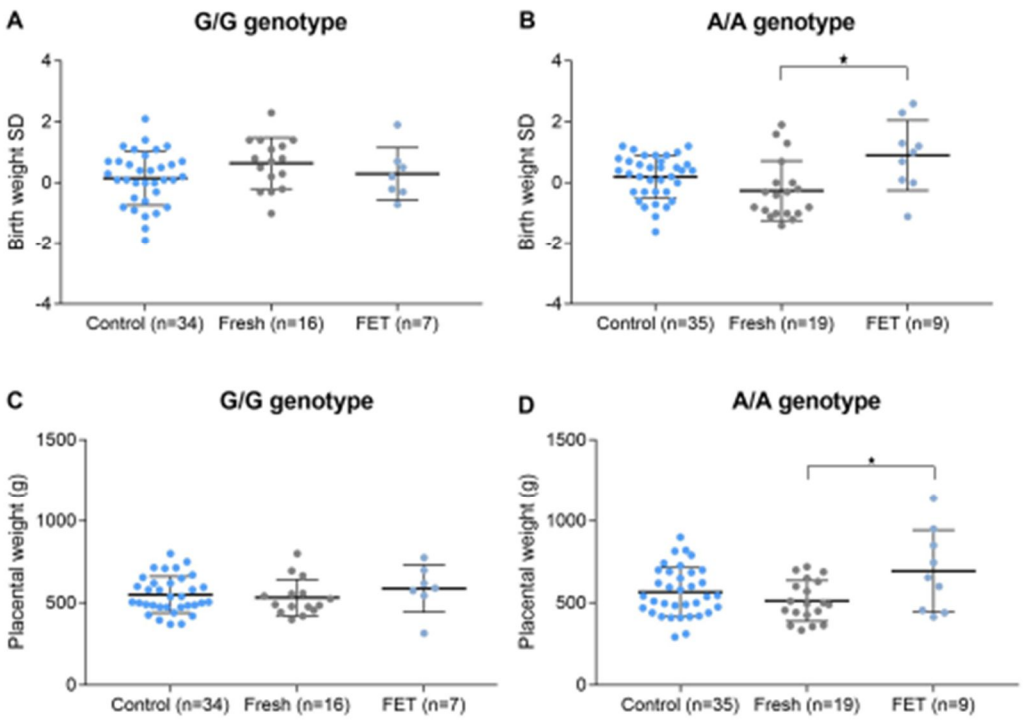


Figure 11. Genotype-specific effects in birth weight (SD) and placental weight of control and fresh and frozen embryo transfer (FET) derived newborns. Birth weights and placental weights differed significantly between the groups (*P*=0.02, *P*=0.006, respectively, One-way ANOVA). **A.** Birth weight SD of G/G genotype **B.** Birth weight SD of A/A genotype. The birth weight of FET derived newborns was higher compared to fresh derived newborns with A/A genotype (*P*=0.02, Bonferroni *post hoc*). **C.** Placental weight (g) of G/G genotype. **D.** Placental weight (g) of A/A genotype. The placental weight of FET derived newborns was higher compared to fresh derived newborns in A/A genotype (*P*=0.004, Bonferroni post hoc). Stars represent: *P < 0.05, **P < 0.01, ***P ≤ 0.001 (One-way ANOVA followed by Bonferroni *post hoc*).

Next, the birth measures of ART and control newborns were examined between all four genotypes. Interestingly, a significant difference was observed in head circumference of ART derived newborns (*P*=0.02, One-way ANOVA). The head circumference of newborns with A/A genotype was smaller compared to newborns with G/G genotype (*P*=0.04, Bonferroni *post hoc*). Significant differences in other birth measures of ART derived newborns between the genotypes were not detected. Moreover, significant differences in any birth measures of controls between the genotypes were not detected.

The birth measures of fresh embryo transfer derived newborns and controls were also examined between all genotypes. The birth measures of FET derived newborns were not compared between the genotypes, due to the low number of heterozygous FET samples. According to results, when comparing the birth weight and head circumference of fresh derived newborns between the genotypes, significant differences were observed (*P*=0.04, *P*=0.004, respectively, One-way ANOVA). Birth weights (*P*=0.04, Bonferroni *post hoc*)

(Figure 12A) and head circumferences (*P*=0.002, Bonferroni *post hoc*) (Figure 12B) were smaller in A/A genotype compared to G/G genotype. Significant differences in other birth measures of fresh derived newborns between the genotypes were not detected. Moreover, significant differences in any birth measures of controls between the genotypes were not detected.

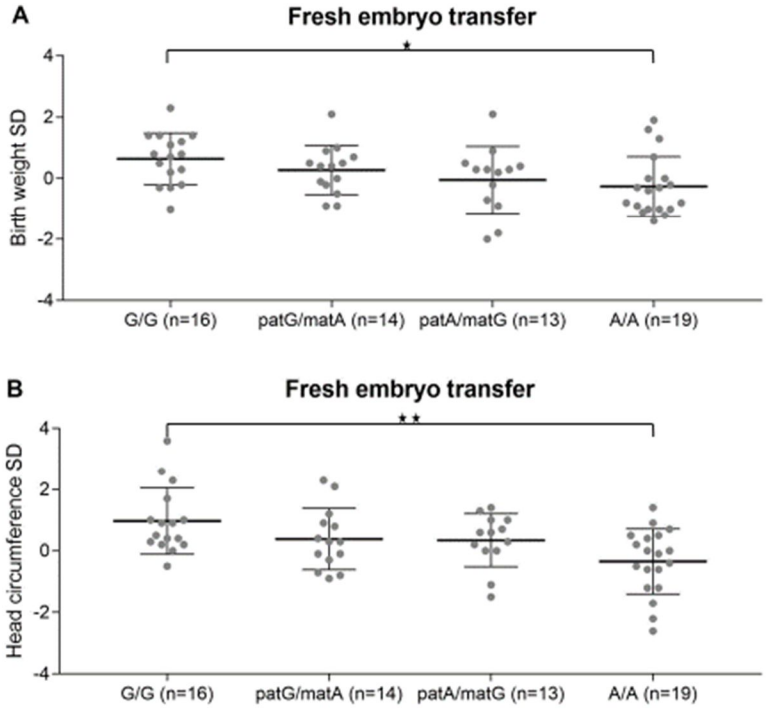


Figure 12. Genotype-specific variation in birth weight and head circumference of fresh embryo transfer (IVF or IVF/ICSI) derived newborns. The birth weight and head circumference of fresh derived newborns differed significantly between the genotypes (*P*=0.04, *P*=0.004, respectively, One-way ANOVA). **A.** Birth weight was smaller in A/A genotype compared to G/G genotype (*P*=0.04, Bonferroni *post hoc*). **B.** The head circumference was smaller in A/A genotype compared to G/G genotype (*P*=0.002, Bonferroni *post hoc*). Stars represent: *P < 0.05, **P < 0.01, ***P ≤ 0.001 (One-way ANOVA followed by Bonferroni *post hoc*).

5 Discussion

5.1 Effects of ART on DNA methylation in the placenta

Since ART procedures take place during the sensitive period of epigenetic reprogramming, this thesis aimed to study whether ART has an impact on DNA methylation in the placenta. Due to the altered birth size of ART newborns, the aim was to explore the methylation levels at CTCF6 of *H19* ICR1 of the *IGF2/H19* imprinted gene locus, important for the growth and

development of the embryo and placenta. Previous studies have shown decreased DNA methylation in the total methylation levels at CTCF6 of *H19* ICR1 in the placenta (Nelissen et al., 2013a), buccal epithelium (Loke et al., 2015) and cord blood mononuclear cells (Castillo-Fernandez et al., 2017) of newborns conceived by IVF or IVF/ICSI.

In this study, no difference in the total methylation levels between ART derived and control placentas was identified. However, according to the bisulfite sequencing analysis, when methylation levels were examined in rs10732516 G/A genotype- and allele-specific manner, altered DNA methylation levels of ART derived placental samples were detected. CTCF6 of *H19* ICR1 of ART derived placentas in the paternal allele of patA/matG genotype was seen to be slightly, but consistently, hypomethylated at several CpG sites compared to placentas of spontaneously conceived newborns. By contrast, the same decrease in methylation levels was not seen in the patG/matA genotype. Thus, the results indicate that the effects of ART on the DNA methylation levels at the imprinted *IGF2/H19* gene locus in the placenta are genotype-specific and dependent on the parent-of-origin. However, the same genotype-specific methylation changes at CTCF6 of *H19* ICR1 in placenta was not seen by the EpiTYPER method. EpiTYPER analysis indicated increased methylation levels at two CpG sites of *H19* DMR in A/A genotype in ART-derived placentas.

When studying the DNA methylation levels at CTCF6 of *H19* ICR1 in white blood cells in cord blood of patA/matG genotype, similar decreased methylation levels in paternal allele, as in ART derived placental samples, were not detected. By contrast, slightly increased methylation levels were observed. Thus, the results suggest that the effects of ART on DNA methylation at *H19* ICR1 are also cell type-specific. This could be explained by the different characteristics between the formation of embryonic and extraembryonic cell types. Above all, in blastocyst stage, the extraembryonic trophectoderm cells, from which the placenta is derived, are directly in contact with the *in vitro* culture, unlike the embryonic inner cell mass from which the fetus is formed. Moreover, the trophoblast cells are at different differentiation stage than inner cell mass and the two cell lines may also have different repair mechanism to correct epigenetic changes. However, the cell type-specific effect of *in vitro* culture on *H19* imprinting has already been seen in previous mouse studies: Mann et al. (2004) demonstrated that placental tissues displayed loss of *H19* imprinted gene expression, while the embryos maintained the proper imprinted gene expression. The loss of imprinted gene expression was also able to associate with decreased DNA methylation at *H19*. The result of this thesis is also consistent with the earlier study on ART newborns, in which decreased

DNA methylation was detected at CTCF6 only in mononuclear, but not in white blood cells (Castillo-Fernandez et al., 2017).

5.2 Effects of ART on the phenotype of newborns

Due to the lower birth weight of IVF and IVF/ICSI newborns derived from fresh embryo transfer and, by contrast, increased birthweight of IVF and IVF/ICSI newborns derived from frozen embryo transfer (FET), this thesis aimed to study the effects of ART on the phenotype of newborns. According to the results, lower birthweights of IVF and IVF/ICSI newborns derived from fresh embryo transfer, compared to spontaneously conceived newborns, were not detected as in previous studies (Doyle et al., 1992; Helmerhorst et al., 2004; Jackson et al., 2004; Malchau et al., 2014). However, the placentas of FET derived newborns were heavier than placentas of fresh embryo transfer derived newborns and control newborns.

The effects of ART on the phenotype of newborns were also examined in rs10732516 G/A genotype-specific manner between ART and control newborns. According to the results, the head circumference (SD) of ART newborns was smaller than controls in A/A genotype, but not in other genotypes. The genotype-specific effects on phenotype were also explored between controls, fresh embryo transfer and FET derived newborns. According to the results, differences in phenotype between the newborns were seen in A/A genotype, but not in G/G genotype. In A/A genotype, FET derived newborns were heavier than fresh embryo transfer derived newborns, which is consistent to earlier findings (Maheshwari et al., 2018; Pelkonen et al., 2010; Wennerholm et al., 2013). Also, in A/A genotype, the placentas of FET newborns were heavier than the placentas of fresh embryo transfer derived newborns. The genotype-specific examination also revealed smaller birth weight and head circumference of fresh embryo transfer derived newborns with A/A genotype than with G/G genotype. Thus, the results of the genotype-specific examination suggest that the effects of ART on the phenotype associates with the rs10732516 polymorphism.

5.3 Potential causalities and further research

The genotype-specific examination of methylation levels and the phenotype of newborns suggests that the effects of ART are rs10732516 G/A genotype-specific and dependent on the parent-of-origin. Interestingly, the results are consistent to the earlier study where

rs10732516 G/A polymorphism and another environmental factor, prenatal alcohol exposure, was seen to associate with decreased methylation levels at the *IGF2/H19* locus and alterations in the phenotype of newborns (Marjonen et al., 2017). A similar decrease in the methylation levels at the paternal allele in rs10732516 patA/matG genotype in alcohol-exposed placentas were discovered as in ART derived placentas in this study. Moreover, increased head circumference was observed in alcohol-exposed newborns only with A/A genotype, which is consistent with the increased birth weight of FET derived newborns, but not with decreased head circumference of fresh embryo transfer derived newborns in that same genotype. Even though ART and early prenatal alcohol exposure both take place during the epigenetic reprogramming in the early embryo and have been associated with intrauterine growth restriction, they are yet very different environmental factors. Although, also other previous studies indicate that the rs10732516 polymorphism associates with the newborn's size at birth: polymorphisms rs4929984 and rs2071094, which both are in linkage to rs10732516, have been previously seen to associate with the birth size (Adkins et al., 2010; Petry et al., 2005, 2011).

The possible different response of the rs10732516 patA/matG and A/A genotypes to the environmental factors could be explained by the underlying DNA sequence. It is known that single nucleotide polymorphisms may affect the methylation levels of nearby CpG sites (Feil & Fraga, 2012) and changes in DNA methylation at binding sites of CTCFs in ICRs are associated with differential CTCF protein binding (Wang et al., 2012). Thus, the rs10732516 G/A polymorphism may affect the binding of CTCF protein, since A allele deletes a CpG binding site for a methyl group. Consequently, the polymorphism and, hence, changed DNA methylation profile could potentially affect the regulation and function of the *IGF2/H19* gene locus. Hypomethylation of *H19* ICR1 in paternal allele of rs10732516 patA/matG genotype, in this study, anticipates the overexpression of *H19*, downregulation of *IGF2,* and hence poor fetal growth and IUGR (Bourque et al., 2010), which have been seen among fresh embryo transfer derived IVF and IVF/ICSI newborns (Doyle et al., 1992; Helmerhorst et al., 2004; Jackson et al., 2004; Malchau et al., 2014). Consistently, the results of this study showed that IVF and IVF/ICSI newborns derived from fresh embryo transfer were smallest in patA/matG and A/A genotypes.

Despite the interesting findings of this study, the association of rs10732516 G/A polymorphism and ART on the regulation of the *IGF2/H19* locus should be further studied. First, the effects of the A allele on the binding of CTCF protein should be confirmed by functional studies. Moreover, whether the genotype-specific changes in DNA methylation

affect the placental gene expression of the *IGF2/H19* locus and, consequently, the growth of the embryo, should be explored. Nonetheless, in addition to *H19* ICR1, the *H19* promoter (*H19* DMR) and *IGF2* DMRs are also crucial for the proper parent-of-origin gene expression of the *IGF2/H19* gene locus. DMRs are suggested to function in the interaction between *H19* ICR1, consequently, affecting the chromatin structure and tissue-specific imprinted gene expression (Muller et al., 2004). Therefore, the regulation of this rather complex imprinted gene locus is not straightforward and needs further investigations.

According to the results of this and previous studies (Maheshwari et al., 2018; Pelkonen et al., 2010; Wennerholm et al., 2013), frozen embryo transfer seems to affect the risk for higher birth weight of newborns conceived by IVF or IVF/ICSI. Hence, further studies are needed to explore the effects of embryo freezing on the phenotype of newborns. It would be also interesting to investigate whether the effects on DNA methylation levels at the *IGF2/H19* gene locus are different between fresh embryo transfer and FET and whether the changes associate with the rs10732516 G/A polymorphism. Moreover, it would be interesting to investigate how the rs10732516 G/A polymorphism affects the further development and growth of the ART children and whether the frequency of imprinting disorders associates with the polymorphism.

Most of all, this study on DNA methylation profiles at the *IGF2/H19* locus, could not reveal whether the changes are due to the ART procedures themselves or underlying infertility. Similar decreased methylation levels at the *IGF2/H19* locus have been detected in sperm of infertile men, especially hypomethylation at the CTCF6 of *H19* ICR1 (Boissonnas et al., 2010; Marques et al., 2008) as in ART newborns with rs10732516 patA/matG genotype in this study. Some studies indicate that the differences in methylation profiles may be related to both, ART procedures and infertility, as observed in the study by Song et al. (2015) with autologous oocytes with parental infertility and donor oocytes without parental infertility. Some studies, however, have observed that infertility itself is related to altered gene expression of placental imprinted genes. Litzky et al. (2017) found several imprinted genes with significantly different placental gene expression between infertile newborns and controls, but no differences between IVF newborns and controls. Still, 4 genes, including *IGF2*, were significantly downregulated in IVF newborns compared to the infertile group, indicating that these genes may be affected by the IVF procedure. In addition to alterations in epigenetic profiles, both ART and infertility has been associated with adverse perinatal outcomes (Basso & Baird, 2003; Luke et al., 2016; Palomba et al., 2016; Seggers et al., 2015). However, studies on singleton siblings from same parents have indicated that singletons,

conceived by IVF, have a poorer outcome than their siblings that have been conceived naturally (Pinborg et al., 2013). Nonetheless, more research is needed to discern the effects of ART and infertility on the changes in epigenetic profiles and the phenotype of newborns.

5.4 Limitations of the study

There are several limitations in this study. First, due to the sample division to four genotypes, more samples are needed to confirm the suggested genotype-specific effects. Moreover, the genotype and allele-specific methylation levels could not be revealed for homozygous A/A and G/G genotypes with traditional bisulfite sequencing, since it was only suitable to detect the genotype- and allele-specific methylation levels for the rs10732516 patA/matG and patG/matA heterozygous samples. Furthermore, due to the low number of heterozygous (patG/matA, patA/matG) FET derived samples, the genotype-specific examination for controls, fresh embryo transfer and FET derived newborns, could only be done for homozygous G/G and A/A genotypes. Also, due to the low number of FET derived samples, the birth measures of FET derived newborns could not be compared between the genotypes.

Secondly, the limitations of this study are associated with the methylation analysis methodology used. Apparent PCR bias was detected in bisulfite sequencing: the amplification of hypomethylated maternal allele was favorited over hypermethylated paternal allele especially in rs10732516 patA/matG genotype. According to Warnecke et al. (1997), PCR amplification may have preferred the unmethylated DNA strand over the methylated DNA strand, due to higher C/G content, since it raises the melting temperature and may, therefore, increase the formation of secondary structures. However, in this case, the differential amplification of the alleles seems to be dependent on the genotype: also, in the study by Coolen et al. (2011) the PCR bias varied according to the genotype, being strongest in the patA/matG genotype. Moreover, similar decreased methylation levels in placentas of newborns with patA/matG genotype were not detected with EpiTYPER as it was with bisulfite sequencing. This indicates that the EpiTYPER method may not be sufficient to explore minor genotype- and allele-specific methylation changes of the *IGF2/H19* imprinted locus or the paternal and maternal alleles were not equally amplified in PCR. Thus, the results of methylation analyses should be confirmed with other methods that are not based on PCR.

6 Conclusions

The first aim of this thesis was to explore the effects of ART on DNA methylation of growth-related *IGF2/H19* imprinted gene locus in the placenta. Consistent to previous ART study (Nelissen et al., 2013a), the result showed consistently decreased DNA methylation at the sixth binding sequence (CTCF6) of *H19* ICR in ART derived placentas. Interestingly, the changes were seen to be genotype-specific and to associate with the rs10732516 G/A polymorphism. Since the *IGF2/H19* imprinted gene locus has important role in the development of the embryo and placenta, these changes may induce alterations in newborn's size at birth in a genotype-specific manner. However, whether these changes in DNA methylation affect the regulation and gene expression of this rather complex imprinted gene locus, should be explored in further studies. In addition to extraembryonic placental cells, this thesis also aimed to study whether similar DNA methylation changes at the *IGF2/H19* gene locus could also be seen in embryonic white blood cells. Similar changes in DNA methylation levels in white blood cells were not observed, which is consistent to the previous studies (Castillo-Fernandez et al., 2017) and suggests that the effects of ART on DNA methylation at the *IGF2/H19* locus are also cell type-specific.

The second aim of this thesis was to explore the effects of ART on the phenotype of newborns. The effects of ART on the phenotype also seemed to be genotype-specific and to associate with the rs10732516 polymorphism. Fresh embryo transfer derived newborns with A/A genotype were seen to have smaller birth weight and head circumference than newborns with G/G genotype. Moreover, in A/A genotype, FET derived newborns were demonstrated to be heavier and to have heavier placentas than fresh embryo transfer derived newborns. Consistently to previous studies (Maheshwari et al., 2018; Pelkonen et al., 2010; Wennerholm et al., 2013), this also indicates that the effects on newborn's birth weight are different between fresh and frozen embryo transfer. Since alterations in birth size are known to have long-term health implications, further studies are needed to reveal the effects of IVF and ICSI procedures with fresh and frozen embryos and the causes of the effects.

The findings of this thesis suggest that ART has effects on DNA methylation in the placenta and on the phenotype of newborns, and the effects associate with the rs10732516 G/A polymorphism. This result underlines the significance of the polymorphism when studying the effects of ART. However, further investigations are needed to confirm these findings and to discern whether the changes are due to the ART procedures or underlying infertility.

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Appendix A. ART sample information

Table 1. Sample information of ART newborns and mothers.

Genotype	Fresh/FET	Treatment	Country	Gender	Gestational weeks+days	5 min Apgar score	Maternal age	Maternal BMI	Weight (g)	Weight SD	Lenght (cm)	Lenght SD	Head circumference (cm)	Head circumference SD	Placetal weight (g)
G/G	Fresh	IVF	Finnish	Male	40+0	10	32	27,0	3700	0,28	51	-0,07	35,5	0,32	800
G/G	Fresh	IVF	Finnish	Female	39+0	9	40	23,0	3100	-0,29	50	0,2	33,5	-0,47	556
G/G	Fresh	IVF	Finnish	Female	38+5	10	26	22,7	2735	-0,99	46	-1,38	34	0	455
G/G	Fresh	IVF	Finnish	Female	39+6	10	28	19,0	3810	0,8	52	0,74	35,8	0,85	695
G/G	Fresh	IVF	Finnish	Female	39+6	9	40	19,5	4015	1,17	52	0,74	37	1,72	665
G/G	Fresh	IVF	Estonian	Male	41+3	9	45	24,0	3786	-0,2	52	-0,2	36	0,17	477
G/G	Fresh	IVF	Estonian	Male	39+2	9			4084	1,39	51	0,22	38	2,32	485
G/G	Fresh	IVF	Estonian	Male	39+5	9	32	29,4	4660	2,31	54	1,37	40	3,58	
G/G	Fresh	IVF	Estonian	Male	38+4	9	35	24,9	3640	0,79	50	0,07	34,5	0,16	421
G/G	Fresh	IVF	Estonian	Female	39+4	9	37	30,1	3444	0,21	50	-0,03	35	0,38	399
G/G	Fresh	IVF	Estonian	Male	39+1	9	26	25,7	4074	1,43	52	0,71	36	0,98	526
G/G	Fresh	IVF	Estonian	Male	39+1	8			3888	1,05	51	0,27	36	0,98	479
G/G	Fresh	IVF/ICSI	Estonian	Male	40+5	9	33	35,1	4088	0,74	52	0,09	36	0,42	558
G/G	Fresh	IVF/ICSI	Estonian	Male	37+5	9	35	30,1	2962	-0,3	48	-0,42	34,5	0,5	443
G/G	Fresh	IVF/ICSI	Estonian	Female	39+4	8	31	30,4	4074	1,37	52	0,84	38,5	2,55	542
G/G	Fresh	IVF/ICSI	Estonian	Male	40+3	9	25	27,2	3906	0,51	52	0,2	36,5	0,87	490
G/G	FET	IVF	Finnish	Female	38+1	10	38	22,0	3340	0,54	51	0,94	36	1,64	700
G/G	FET	IVF	Finnish	Male	41+3	9	38	17,3	3744	-0,28	53	0,26	36,5	0,53	545
G/G	FET	IVF	Finnish	Male	40+4	10	40	21,0	3600	-0,19	50	-0,76	36	0,47	575
G/G	FET	IVF	Finnish	Female	39+6	9	36	30,3	3470	0,16	49	-0,57	34	-0,45	590
G/G	FET	IVF	Finnish	Female	40+5	10	39	26,3	3930	0,72	48	-1,37	34	-0,78	618
G/G	FET	IVF	Estonian	Female	41+3	9	34	36,7	4786	1,88	54	1,08	35	-0,3	775
G/G	FET	IVF/ICSI	Estonian	Male	34+6	9	44	31,1	2163	-0,7	45	-0,33	31	-0,54	316
patG/matA	Fresh	IVF	Finnish	Male	39+0	9	42	24,0	2970	-0,87	49	-0,54	33,5	-0,7	465
patG/matA	Fresh	IVF	Finnish	Female	40+3	10	36	22,7	3240	-0,51	50	-0,36	36	0,8	520
patG/matA	Fresh	IVF	Finnish	Female	42+0	9	32	20,6	4350	0,99	53	0,41	34,5	-0,87	625
patG/matA	Fresh	IVF	Finnish	Female	40+5	9	41	23,7	3500	-0,08	51	-0,02	34	-0,78	677
patG/matA	Fresh	IVF	Estonian	Female	40+3	10	30	30,0	3724	0,45	52	0,53	35,5	0,43	512
patG/matA	Fresh	IVF	Estonian	Female	37+0	9	40	29,7	3278	0,92	50	0,97	36	2,11	411
patG/matA	Fresh	IVF	Estonian	Male	38+3	9	39	23,2	2858	-0,87	50	0,13	34	-0,13	440
patG/matA	Fresh	IVF	Estonian	Female	40+6	9	36	28,4	3790	0,42	51	-0,08	35	-0,09	525
patG/matA	Fresh	IVF	Estonian	Male	39+2	9	29	32,0	3662	0,52	50	-0,22	38	2,32	436
patG/matA	Fresh	IVF	Estonian	Male	39+1	9	31	33,9	3314	-0,17	47	-1,47	35	0,29	342
patG/matA	Fresh	IVF/ICSI	Finnish	Male	37+4	9	34	26,0	4038	2,07	51	0,9	35,5	1,24	670
patG/matA	Fresh	IVF/ICSI	Finnish	Female	38+0	9	31	21,0	3049	0	49	0,17	34	0,3	542
patG/matA	Fresh	IVF/ICSI	Estonian	Male	40+3	9	29	29,7	3988	0,67	52	0,2	36,5	0,87	453
patG/matA	Fresh	IVF/ICSI	Estonian	Female	39+3	10	43	32,1	3528	0,42	50	0,03	34	-0,28	359
patG/matA	FET	IVF	Finnish	Male	40+6	9	36	26,0	3935	0,37	51	-0,42	37	1,08	678
patG/matA	FET	IVF	Estonian	Female	37+0	8			2968	0,29	48	0,17	34,5	1,08	415
patG/matA	FET	IVF/ICSI	Finnish	Male	39+0	9	35	25,0	3670	0,66	51	0,33	39	3,11	600
patG/matA	FET	IVF/ICSI	Estonian	Female	42+1	9	34	26,3	3880	0,12	54	0,82	37,5	1,34	590
patA/matG	Fresh	IVF	Finnish	Female	40+1	8	31	22,8	2640	-1,77	48	-1,13	35	0,17	500
patA/matG	Fresh	IVF	Finnish	Male	40+2	9	41	19,8	3874	0,51	52	0,26	36	0,57	505
patA/matG	Fresh	IVF	Finnish	Female	42+0	9	35	20,1	3975	0,34	50	-0,98	37	1,01	750
patA/matG	Fresh	IVF	Finnish	Female	39+5	9	36	21,0	3050	-0,66	49	-0,52	33,5	-1,12	443
patA/matG	Fresh	IVF	Estonian	Male	34+5	7	32	24,4	1586	-2	41,5	-1,66	29,5	-1,47	161
patA/matG	Fresh	IVF	Estonian	Male	39+0	10	41	27,2	3494	0,29	51	0,33	36	1,03	494
patA/matG	Fresh	IVF/ICSI	Finnish	Male	40+1	9	35	24,0	3720	0,26	52	0,32	36	0,62	640
patA/matG	Fresh	IVF/ICSI	Finnish	Male	41+0	9	35	23,0	3365	-0,89	51	-0,48	35,5	-0,04	435
patA/matG	Fresh	IVF/ICSI	Finnish	Male	39+2	9	29	27,0	3624	0,44	48	-1,1	36,5	1,28	630
patA/matG	Fresh	IVF/ICSI	Estonian	Male	40+6	9	37	25,6	3680	-0,15	52	0,03	36,5	0,73	500
patA/matG	Fresh	IVF/ICSI	Estonian	Female	39+5	9	40	23,3	3848	0,92	50	-0,08	35	0,33	411
patA/matG	Fresh	IVF/ICSI	Estonian	Female	40+4	9	39	42,4	3592	0,15	50	-0,42	35	0,01	523
patA/matG	Fresh	IVF/ICSI	Estonian	Male	38+6	8	27	24,9	4326	2,06	54	1,69	36,5	1,43	618
patA/matG	FET	IVF	Finnish	Male	39+6	9	38	18,8	3320	-0,47	50	-0,46	34,5	-0,33	695
patA/matG	FET	IVF	Finnish	Male	39+4	9	34	27,3	4180	1,45	52	0,54	37	1,52	830
patA/matG	FET	IVF	Finnish	Male	41+6	10	34	24,0	4085	0,2	51	-0,84	35	-0,7	747
patA/matG	FET	IVF	Estonian	Male	39+2	10	31	26,6	3818	0,84	52	0,66	35,5	0,58	476
A/A	Fresh	IVF	Finnish	Male	39+3	9	32	21,0	2970	-1,07	47	-1,6	33	-1,22	455
A/A	Fresh	IVF	Finnish	Female	41+2	9	35	33,5	3645	-0,01	49	-1,16	33	-1,73	700
A/A	Fresh	IVF	Finnish	Male	41+2	10	38	19,0	3865	0,03	51	-0,6	36	0,22	650
A/A	Fresh	IVF	Finnish	Female	36+5	9	36	20,0	2194	-1,36	44	-1,29	32	-0,52	495
A/A	Fresh	IVF	Finnish	Male	41+6	10	35	23,0	3550	-0,88	51	-0,84	36	0,02	450
A/A	Fresh	IVF	Finnish	Male	41+6	10	37	21,2	3420	-1,16	52	-0,38	33	-2,15	510
A/A	Fresh	IVF	Finnish	Male	39+4	10	33	19,5	4080	1,25	53	0,99	34	-0,57	720
A/A	Fresh	IVF	Finnish	Female	40+4	9	33	30,0	3144	-0,77	51	0,03	34,5	-0,36	600
A/A	Fresh	IVF	Finnish	Female	40+4	9	36	21,6	3875	0,67	52	0,48	35	0,01	630
A/A	Fresh	IVF	Finnish	Female	40+6	9	35	22,0	3420	-0,3	52	0,37	36	0,65	490
A/A	Fresh	IVF	Estonian	Male	38+5	10	31	26,6	2968	-0,75	51	0,45	33,5	-0,59	362
A/A	Fresh	IVF	Estonian	Male	38+6	8	35	28,7	3134	-0,44	50	-0,05	35	0,39	334
A/A	Fresh	IVF	Estonian	Female	41+2	9	31	26,9	3520	-0,25	50	-0,7	36	0,5	357
A/A	Fresh	IVF	Estonian	Female	39+0	10	45	29,9	3150	-0,19	49,5	-0,02	34	-0,11	443
A/A	Fresh	IVF	Estonian	Male	41+1	9	38	26,8	3366	-0,95	50	-1	34	-1,16	427
A/A	Fresh	IVF	Estonian	Male	38+3	8	31	25,8	2782	-1,04	50	0,13	35,5	0,9	364
A/A	Fresh	IVF/ICSI	Finnish	Female	39+6	10	29	25,0	2942	-0,95	45	-2,33	31	-2,63	500
A/A	Fresh	IVF/ICSI	Estonian	Male	38+1	8			3914	1,55	52	1,1	36	1,35	571
A/A	Fresh	IVF/ICSI	Estonian	Female	40+2	8			4552	1,91	53	1,03	35,5	0,48	689
A/A	FET	IVF	Finnish	Male	39+3	9	36	26,9	3900	0,95	53	1,04	38	2,27	745
A/A	FET	IVF	Finnish	Male	39+4	9	31	26,1	4095	1,28	52	0,54	35	0,13	850
A/A	FET	IVF	Finnish	Female	42+1	9	32	20,0	3818	0	50	-1,04	33,5	-1,68	656
A/A	FET	IVF/ICSI	Finnish	Male	41+2	10	32	19,0	3905	0,11	51	-0,6	36,5	0,58	440
A/A	FET	IVF/ICSI	Finnish	Male	39+4	10	30	21,5	4610	2,28	54	1,43	37	1,52	950
A/A	FET	IVF/ICSI	Finnish	Male	40+0	10	35	22,0	3910	0,71	52	0,38	34,5	-0,38	600
A/A	FET	IVF/ICSI	Finnish	Female	39+3	9	38	28,0	4822	2,62	53	1,33	36,6	1,59	1140
A/A	FET	IVF/ICSI	Finnish	Female	39+6	10	40	26,2	2900	-1,05	48	-1,01	31	-2,63	415
A/A	FET	IVF/ICSI	Estonian	Female	41+6	10	39	26,5	4464	1,23	54	0,93	36	0,3	455

Appendix B. PCR primers and protocols

Table 1. PCR primers. Modified from Marjonen et al., submitted.

Genotyping by sequencing primers

Target	Forward/revese primer	Sequence 5´- 3´	Size (bp)	From publication
H19 ICR1 (CTCF6)	Forward	GGCTTCTCCTTCGGTCTCAC	241	Marjonen et al. 2017
	Reverse	TGTGGATAATGCCCGACCTG		

Bisulfite sequencing primers

Target	Forward/revese primer	Sequence 5´- 3´	Size (bp)	From publication
H19 ICR1 (CTCF6)	Forward	AGGAAGAGAGGGAAAATGTAAGATTTTGGTGGAATAT	465	Coolen et al. 2007
	Reverse	CAGTAATACGACTCACTATAGGGAGAAGGCTTCCCAATTCCATAAATAATAAAAATCTC		

EpiTYPER primers

Target	Forward/revese primer	Sequence 5´- 3´	Size (bp)	From publication
H19 DMR	Forward	AGGAAGAGAGTTTTTATTAAAGGTTAAGGTGGTGAT	255	Ollikainen et al., 2010
	Reverse	CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAACAAAATCCCCACAACC		
H19 ICR1 (CTCF6)	Forward	AGGAAGAGAGTATGGGTATTTTTGGAGGTTTTTTT	316	Ollikainen et al. 2010
	Reverse	CAGTAATACGACTCACTATAGGGAGAAGGCTAACTTAAATCCCAAACCATAACACT		
LINE-1	Forward	AGGAAGAGAGGTGTGAGGTGTTAGTGTGTTTTGTT	433	Wang et al. 2010
	Reverse	CAGTAATACGACTCACTATAGGGAGAAGGCTATATCCCACACCTAACTCAAAAAAT		

Table 2. PCR protocols. Modified from Marjonen et al., submitted.

Genotyping by sequencing PCR protocol

	95° 15min
40x	95° 1 min
	62° 30s
	72° 30s
	72° 10min
	4°∞

Bisulfite sequencing PCR protocol

	95° 15min
40x	95° 1min
	56° 30s
	72° 30s
	72° 10min
	4° ∞

EpiTYPER PCR protocol

	95° 15min
5x	94° 1min
	56° 30s
	72° 30s
40x	94° 1min
	30s 60° for *H19* DMR; 58° for *H19* CTCF6; 56° for LINE-1
	72° 30s
	72° 10min
	4° ∞

Appendix C. Ligation and transformation protocol

1. Set up ligation reactions in sterile 0,5 ml tubes:

Buffer x2	5 µl
Vector	1 µl
PCR product	3 µl
Ligase	1 µl
Total	10 µl

Final volume should be made up to 10 with milli-Q water. Molar ratio between insert and vector should be between 3:1 to 1:3.

2. Incubate overnight at 4°C.
3. Next day, equilibrate ampicillin (100 mg/ml), IPTG (Bioline) (4 mg/ml) and X-gal (Promega) (0,225 mg/ml) Lysogeny broth plates to room temperature. Dry at 37°C for few hours.
4. Add 10 µl of the reactions to a sterile 1,5 ml tube containing 100 µl of just thawed competent cells.
5. Incubate on ice for 20 min.
6. Heat shock for 50 sec. at 42°C exactly and them place on ice for 2 min.
7. Add 950 µl of room temperature luria broth to the tubes and incubate in a 37°C shaking for 1,5 h.
8. Plate 300 µl of each trasformation cultre onto the antibiotic plates.
9. Incubate at 37°C overnight lid up.

Table 1. Total DNA methylation levels and standard deviations at CTCF6 at *H19* ICR1, *H19* DMR and LINE-1 in control and ART placentas by EpiTYPER method. Blue boxes represent significantly changed DNA methylation level at CpG units between ART and control samples (nominal p-value<0,05, Mann-Whitney U). Modified from Marjonen et al., submitted.

H19 ICR1 (CTCF6)

			CpG1	CpG5,6,7,8	CpG10	CpG11,12	CpG13,14	CpG21,22	CpG23
TOTAL	N = 60	Control	0.34 ± 0.03 N=59	0.28 ± 0.04 N=60		0.32 ± 0.03 N=60	0.32 ± 0.03 N=60	0.52 ± 0.06 N=60	0.63 ± 0.08 N=60
	N = 62	ART	0.35 ± 0.04 N=61	0.29 ± 0.04 N=61		0.33 ± 0.03 N=61	0.33 ± 0.03 N=61	0.53 ± 0.07 N=61	0.64 ± 0.08 N=61
G/G	N = 15	Control	0.31 ± 0.02 N=15	0.32 ± 0.04 N=15	0.36 ± 0.02 N=15	0.31 ± 0.02 N=15	0.32 ± 0.02 N=15	0.46 ± 0.03 N=15	0.56 ± 0.04 N=15
	N = 18	ART	0.32 ± 0.04 N=18	0.31 ± 0.03 N=18	0.37 ± 0.03 N=18	0.32 ± 0.03 N=18	0.32 ± 0.02 N=18	0.47 ± 0.05 N=18	0.57 ± 0.05 N=18
A	N = 15	Control	0.34 ± 0.02 N=14	0.30 ± 0.03 N=15	0.82 ± 0.03 N=15	0.32 ± 0.01 N=15	0.31 ± 0.01 N=15	0.58 ± 0.02 N=15	0.70 ± 0.03 N=15
	N = 15	ART	0.36 ± 0.04 N=15	0.31 ± 0.04 N=15	0.82 ± 0.03 N=15	0.34 ± 0.04 N=15	0.33 ± 0.04 N=15	0.59 ± 0.04 N=15	0.70 ± 0.05 N=15
G	N = 15	Control	0.32 ± 0.02 N=15	0.26 ± 0.02 N=15		0.33 ± 0.02 N=15	0.33 ± 0.03 N=15	0.48 ± 0.03 N=15	0.57 ± 0.04 N=15
	N = 14	ART	0.36 ± 0.03 N=14	0.25 ± 0.02 N=14		0.34 ± 0.03 N=14	0.33 ± 0.02 N=14	0.49 ± 0.03 N=14	0.57 ± 0.04 N=14
A/A	N = 15	Control	0.35 ± 0.04 N=15	0.23 ± 0.04 N=15		0.33 ± 0.05 N=15	0.33 ± 0.04 N=15	0.57 ± 0.05 N=15	0.70 ± 0.07 N=15
	N = 15	ART	0.33 ± 0.02 N=14	0.26 ± 0.03 N=14		0.34 ± 0.03 N=14	0.33 ± 0.03 N=14	0.59 ± 0.02 N=14	0.71 ± 0.04 N=14

H19 DMR

			CpG3	CpG6	CpG7,8	CpG9	CpG10	CpG14	CpG16	CpG17,18	CpG20
TOTAL	N = 58	Control	0.33 ± 0.02 N=58	0.26 ± 0.04 N=58	0.27 ± 0.03 N=58	0.32 ± 0.02 N=58	0.34 ± 0.04 N=58	0.31 ± 0.04 N=58	0.31 ± 0.04 N=58	0.27 ± 0.05 N=58	0.29 ± 0.05 N=58
	N = 57	ART	0.34 ± 0.02 N=56	0.27 ± 0.04 N=56	0.28 ± 0.03 N=56	0.32 ± 0.02 N=57	0.35 ± 0.04 N=54	0.33 ± 0.07 N=57	0.33 ± 0.04 N=57	0.28 ± 0.04 N=57	0.29 ± 0.06 N=57
G/G	N = 15	Control	0.32 ± 0.02 N=13	0.26 ± 0.03 N=13	0.27 ± 0.03 N=13	0.32 ± 0.02 N=13	0.35 ± 0.03 N=13	0.31 ± 0.04 N=13	0.32 ± 0.03 N=13	0.28 ± 0.04 N=13	0.30 ± 0.04 N=13
	N = 18	ART	0.34 ± 0.02 N=18	0.27 ± 0.03 N=18	0.28 ± 0.03 N=18	0.32 ± 0.02 N=18	0.35 ± 0.03 N=15	0.33 ± 0.06 N=18	0.33 ± 0.03 N=18	0.28 ± 0.03 N=18	0.29 ± 0.05 N=18
A	N = 15	Control	0.34 ± 0.01 N=15	0.26 ± 0.03 N=15	0.28 ± 0.03 N=15	0.32 ± 0.02 N=15	0.36 ± 0.43 N=15	0.32 ± 0.04 N=15	0.33 ± 0.03 N=15	0.29 ± 0.04 N=15	0.30 ± 0.04 N=15
	N = 15	ART	0.35 ± 0.02 N=12	0.26 ± 0.03 N=12	0.27 ± 0.03 N=12	0.33 ± 0.03 N=13	0.34 ± 0.04 N=13	0.34 ± 0.04 N=13	0.32 ± 0.04 N=13	0.27 ± 0.05 N=13	0.28 ± 0.06 N=13
G	N = 15	Control	0.33 ± 0.02 N=15	0.26 ± 0.05 N=15	0.26 ± 0.04 N=15	0.31 ± 0.02 N=15	0.34 ± 0.04 N=15	0.30 ± 0.04 N=15	0.31 ± 0.05 N=15	0.26 ± 0.07 N=15	0.28 ± 0.06 N=15
	N = 12	ART	0.34 ± 0.02 N=12	0.27 ± 0.04 N=12	0.28 ± 0.03 N=12	0.33 ± 0.02 N=12	0.34 ± 0.03 N=12	0.34 ± 0.11 N=12	0.33 ± 0.05 N=12	0.28 ± 0.06 N=12	0.29 ± 0.06 N=12
A/A	N = 15	Control	0.33 ± 0.02 N=15	0.25 ± 0.04 N=15	0.26 ± 0.03 N=15	0.31 ± 0.03 N=15	0.33 ± 0.04 N=15	0.29 ± 0.04 N=15	0.30 ± 0.03 N=15	0.26 ± 0.03 N=15	0.27 ± 0.04 N=15
	N = 15	ART	0.35 ± 0.02 N=14	0.27 ± 0.04 N=14	0.28 ± 0.03 N=14	0.34 ± 0.03 N=14	0.35 ± 0.05 N=14	0.30 ± 0.04 N=14	0.33 ± 0.05 N=14	0.28 ± 0.05 N=14	0.29 ± 0.07 N=14

LINE-1

			CpG1	CpG2	CpG3	CpG5	CpG6,7	CpG8,9	CpG11,12
TOTAL	N = 16	Control	0.23 ± 0.03 N=16	0.24 ± 0.01 N=16	0.30 ± 0.02 N=16	0.04 ± 0.02 N=16	0.19 ± 0.06 N=16	0.19 ± 0.04 N=16	0.32 ± 0.03 N=16
	N = 16	ART	0.23 ± 0.03 N=16	0.23 ± 0.02 N=16	0.30 ± 0.02 N=16	0.04 ± 0.01 N=16	0.22 ± 0.04 N=16	0.20 ± 0.02 N=16	0.31 ± 0.02 N=16

Table 1. DNA methylation levels of placental samples. Total, genotype- (rs10732516) and allele-specific DNA methylation levels and standard deviations at CTCF6 of *H19* ICR1 of control and ART placentas by traditional bisulfite sequencing. Blue boxes represent significantly changed DNA methylation level at CpG sites between control and ART placentas. Bold numbers represent significantly changed methylation levels at CpG sites between alleles within control or ART samples (nominal P-value<0,05, Mann-Whitney U). Modified from Marjonen et al., submitted.

Total methylation levels

Total	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	12	535	0,51 ± 0,07	0,51 ± 0,06	0,51 ± 0,06	0,49 ± 0,06	0,51 ± 0,04	0,48 ± 0,09	0,51 ± 0,05	0,50 ± 0,05	0,49 ± 0,07	0,25 ± 0,24	0,50 ± 0,05	0,51 ± 0,04	0,51 ± 0,03	0,51 ± 0,04	0,51 ± 0,05	0,48 ± 0,05	0,50 ± 0,06	0,48 ± 0,04	0,50 ± 0,04	0,51 ± 0,03	0,50 ± 0,05	0,48 ± 0,04	0,47 ± 0,06	0,54 ± 0,06	0,70 ± 0,11	0,68 ± 0,08	0,63 ± 0,06
ART placenta	14	680	0,45 ± 0,08	0,46 ± 0,07	0,41 ± 0,08	0,45 ± 0,06	0,46 ± 0,06	0,44 ± 0,08	0,47 ± 0,04	0,48 ± 0,06	0,47 ± 0,07	0,24 ± 0,23	0,48 ± 0,04	0,47 ± 0,03	0,48 ± 0,04	0,47 ± 0,04	0,47 ± 0,06	0,45 ± 0,03	0,48 ± 0,05	0,48 ± 0,05	0,48 ± 0,04	0,45 ± 0,08	0,47 ± 0,03	0,44 ± 0,05	0,42 ± 0,07	0,50 ± 0,09	0,70 ± 0,12	0,67 ± 0,07	0,59 ± 0,06

Genotype-specific methylation levels

Total	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	285	0,50 ± 0,05	0,52 ± 0,06	0,50 ± 0,06	0,49 ± 0,05	0,50 ± 0,03	0,49 ± 0,04	0,50 ± 0,02	0,51 ± 0,02	0,47 ± 0,03	0,48 ± 0,02	0,50 ± 0,01	0,49 ± 0,02	0,51 ± 0,03	0,50 ± 0,03	0,50 ± 0,03	0,47 ± 0,02	0,50 ± 0,03	0,48 ± 0,02	0,49 ± 0,02	0,50 ± 0,02	0,49 ± 0,02	0,47 ± 0,03	0,47 ± 0,04	0,55 ± 0,08	0,78 ± 0,08	0,75 ± 0,02	0,65 ± 0,06
ART placenta	6	274	0,50 ± 0,09	0,49 ± 0,07	0,46 ± 0,06	0,49 ± 0,04	0,50 ± 0,05	0,48 ± 0,05	0,47 ± 0,03	0,49 ± 0,03	0,47 ± 0,07	0,46 ± 0,03	0,48 ± 0,03	0,47 ± 0,03	0,49 ± 0,03	0,48 ± 0,03	0,48 ± 0,03	0,45 ± 0,02	0,49 ± 0,02	0,49 ± 0,04	0,49 ± 0,02	0,46 ± 0,04	0,47 ± 0,03	0,45 ± 0,04	0,43 ± 0,03	0,57 ± 0,06	0,79 ± 0,07	0,69 ± 0,05	0,60 ± 0,09

patA/matG

Total	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	250	0,52 ± 0,08	0,51 ± 0,06	0,52 ± 0,06	0,49 ± 0,07	0,51 ± 0,05	0,46 ± 0,12	0,53 ± 0,07	0,50 ± 0,06	0,50 ± 0,09	0,03 ± 0,05	0,50 ± 0,08	0,52 ± 0,05	0,51 ± 0,03	0,51 ± 0,05	0,51 ± 0,07	0,49 ± 0,08	0,50 ± 0,08	0,47 ± 0,05	0,52 ± 0,05	0,52 ± 0,03	0,51 ± 0,07	0,48 ± 0,05	0,48 ± 0,09	0,53 ± 0,05	0,61 ± 0,07	0,61 ± 0,05	0,60 ± 0,05
ART placenta	8	406	0,41 ± 0,05	0,42 ± 0,05	0,36 ± 0,05	0,42 ± 0,06	0,42 ± 0,06	0,40 ± 0,08	0,48 ± 0,05	0,48 ± 0,08	0,47 ± 0,07	0,01 ± 0,02	0,48 ± 0,05	0,48 ± 0,03	0,48 ± 0,05	0,45 ± 0,04	0,45 ± 0,08	0,46 ± 0,04	0,47 ± 0,06	0,47 ± 0,06	0,47 ± 0,05	0,44 ± 0,10	0,47 ± 0,04	0,44 ± 0,06	0,40 ± 0,08	0,44 ± 0,07	0,60 ± 0,05	0,64 ± 0,07	0,58 ± 0,04

Allele-specific methylation levels

Paternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	169	0,93 ± 0,03	0,94 ± 0,05	**0,90 ± 0,05**	0,93 ± 0,05	0,95 ± 0,04	0,93 ± 0,06	0,95 ± 0,03	0,96 ± 0,03	0,92 ± 0,08	0,95 ± 0,04	0,95 ± 0,04	0,96 ± 0,03	0,96 ± 0,04	0,95 ± 0,05	0,97 ± 0,03	0,90 ± 0,06	0,96 ± 0,05	0,93 ± 0,04	0,95 ± 0,05	**0,96 ± 0,03**	0,95 ± 0,04	0,89 ± 0,03	0,89 ± 0,03	0,93 ± 0,03	0,97 ± 0,03	0,98 ± 0,03	0,91 ± 0,04
ART placenta	6	128	0,90 ± 0,12	**0,91 ± 0,08**	**0,86 ± 0,09**	**0,93 ± 0,07**	**0,93 ± 0,07**	0,92 ± 0,09	0,92 ±0,07	0,97 0,04	0,93 ± 0,14	0,92 ± 0,06	0,94 ± 0,07	0,93 ± 0,06	0,96 ± 0,04	**0,95 ± 0,05**	0,95 ± 0,05	0,88 ± 0,03	0,97 ± 0,04	0,94 ± 0,04	0,98 ± 0,03	0,92 ± 0,09	0,93 ± 0,05	0,88 ± 0,05	0,85 ± 0,09	**0,98 ± 0,02**	**0,98 ± 0,03**	0,91 ± 0,04	0,92 ± 0,07

Maternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	116	0,08 ± 0,08	0,10 ± 0,10	0,10 ± 0,10	0,05 ± 0,08	0,05 ± 0,08	0,06 ± 0,07	0,04 ± 0,05	0,05 ± 0,05	0,03 ± 0,03		0,05 ± 0,05	0,02 ± 0,05	0,06 ± 0,06	0,05 ± 0,05	0,03 ± 0,05	0,03 ± 0,04	0,04 ± 0,05	0,04 ± 0,05	0,03 ± 0,04	0,03 ± 0,04	0,03 ± 0,04	0,04 ± 0,05	0,04 ± 0,05	0,18 ± 0,14	**0,59 ± 0,15**	**0,53 ± 0,06**	0,39 ± 0,12
ART placenta	6	146	0,09 ± 0,10	0,07 ± 0,07	0,07 ± 0,07	0,05 ± 0,04	0,06 ± 0,04	0,04 ± 0,04	0,02 ± 0,03	0,01 ± 0,01	0,01 ± 0,02		0,01 ± 0,02	0,02 ± 0,02	0,01 ± 0,02	0,02 ± 0,03	0,01 ± 0,01	0,01 ± 0,02	0,01 ± 0,02	0,03 ± 0,04	0,01 ± 0,01	0,01 ± 0,01	0,01 ± 0,03	0,02 ± 0,03	0,02 ± 0,03	0,15 ± 0,11	**0,61 ± 0,13**	0,47 ± 0,11	0,29 ± 0,17

patA/matG

Paternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	42	0,94 ± 0,10	0,93 ± 0,08	**0,98 ± 0,04**	0,93 0,09	0,97 ± 0,07	0,85 ± 0,17	0,98 ± 0,04	0,94 ± 0,08	0,94 ± 0,11		0,95 ± 0,08	0,98 ± 0,04	0,98 ± 0,04	0,98 ± 0,04	0,97 ± 0,07	0,94 ± 0,10	0,94 ± 0,10	0,89 ± 0,10	0,98 ± 0,04	**1,00 ± 0,00**	0,97 ± 0,07	0,91 ± 0,11	0,91 ± 0,11	0,97 ± 0,05	0,95 ± 0,06	0,97 ± 0,07	0,89 ± 0,10
ART placenta	8	84	0,75 ± 0,12	**0,78 ± 0,10**	**0,66 ± 0,13**	**0,78 ± 0,13**	**0,79 ± 0,12**	0,73 ± 0,19	0,94 ± 0,08	0,93 ± 0,14	0,92 ± 0,13		0,94 ± 0,08	0,92 ± 0,07	0,94 ± 0,08	**0,87 ± 0,05**	0,88 ± 0,14	0,89 ± 0,10	0,91 ± 0,10	0,91 ± 0,10	0,91 ± 0,07	0,87 ± 0,18	0,91 ± 0,08	0,81 ± 0,09	0,76 ± 0,17	**0,78 ± 0,14**	**0,85 ± 0,07**	0,89 ± 0,06	0,81 ± 0,09

Maternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control placenta	6	208	0,09 ± 0,11	0,08 ± 0,10	0,06 ± 0,10	0,06 ± 0,09	0,05 ± 0,06	0,06 ± 0,12	0,07 ± 0,12	0,06 ± 0,10	0,06 ± 0,11	0,06 ± 0,10	0,04 ± 0,11	0,05 ± 0,08	0,03 ± 0,04	0,05 ± 0,078	0,06 ± 0,10	0,05 ± 0,08	0,05 ± 0,09	0,04 ± 0,07	0,05 ± 0,08	0,04 ± 0,06	0,06 ± 0,11	0,06 ± 0,08	0,04 ± 0,10	0,08 ± 0,07	**0,27 ± 0,09**	**0,24 ± 0,06**	0,32 ± 0,05
ART placenta	8	322	0,06 ± 0,06	0,06 ± 0,07	0,06 ± 0,07	0,05 ± 0,05	0,05 ± 0,06	0,07 ± 0,07	0,02 ± 0,04	0,02 ± 0,05	0,03 ± 0,06	0,02 ± 0,04	0,03 ± 0,05	0,03 ± 0,05	0,03 ± 0,06	0,03 ± 0,05	0,02 ± 0,05	0,03 ± 0,04	0,02 ± 0,05	0,02 ± 0,05	0,02 ± 0,05	0,02 ± 0,05	0,03 ± 0,06	0,06 ± 0,06	0,04 ± 0,04	0,10 ± 0,07	**0,35 ± 0,12**	0,39 ± 0,10	0,34 ± 0,08

*rs10732516

Table 2. DNA methylation levels of WBC samples. Allele-specific DNA methylation levels and standards deviations at CTCF6 of *H19* ICR1 of control and ART WBCs within rs10732516 patA/matG genotype by traditional bisulfite sequencing. Modified from Marjonen et al., submitted.

Allele-specific methylation levels

patA/matG

Paternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control WBC	4	54	0,71 ± 0,15	0,78 ± 0,10	0,78 ± 0,10	0,80 ± 0,03	0,76 ± 0,10	0,77 ± 0,10	0,95 ± 0,04	0,94 ± 0,04	0,90 ± 0,11		0,94 ± 0,09	0,93 ± 0,09	0,96 ± 0,05	0,98 ± 0,03	0,98 ± 0,03	0,93 ± 0,06	0,92 ± 0,07	0,95 ± 0,06	0,89 ± 0,05	0,97 ± 0,04	0,97 ± 0,04	0,87 ± 0,03	0,86 ± 0,04	0,84 ± 0,08	0,89 ± 0,02	0,86 ± 0,04	0,83 ± 0,03
ART WBC	4	50	0,86 ± 0,04	0,91 ± 0,07	0,89 ± 0,08	0,91 ± 0,07	0,92 ± 0,10	0,92 ± 0,17	1,00 ± 0,00	0,98 ± 0,03	1,00 ± 0,00		1,00 ± 0,00	0,96 ± 0,04	0,93 ± 0,07	1,00 ± 0,00	0,98 ± 0,03	0,92 ± 0,06	0,93 ± 0,07	0,98 ± 0,03	0,96 ± 0,04	0,95 ± 0,06	0,94 ± 0,07	0,89 ± 0,12	0,95 ± 0,06	0,93 ± 0,06	0,93 ± 0,09	0,93 ± 0,06	0,88 ± 0,11

Maternal allele	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control WBC	4	232	0,04 ± 0,01	0,04 ± 0,02	0,03 ± 0,02	0,05 ± 0,03	0,04 ± 0,02	0,04 ± 0,02	0,01 ± 0,02	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,03	0,00 ± 0,00	0,00 ± 0,01	0,00 ± 0,00	0,00 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,11 ± 0,06	0,24 ± 0,08	0,55 ± 0,10	0,61 ± 0,09
ART WBC	4	231	0,04 ± 0,04	0,05 ± 0,05	0,04 ± 0,04	0,04 ± 0,04	0,04 ± 0,04	0,04 ± 0,04	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,01	0,00 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,01	0,00 ± 0,00	0,07 ± 0,01	0,21 ± 0,09	0,52 ± 0,05	0,63 ± 0,15

Total	N	N of clones	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10*	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG18	CpG19	CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	CpG27
Control WBC	4	286	0,37 ± 0,08	0,41 ± 0,05	0,41 ± 0,05	0,42 ± 0,02	0,40 ± 0,06	0,40 ± 0,05	0,48 ± 0,03	0,47 ± 0,02	0,45 ± 0,05	0,00 ± 0,00	0,47 ± 0,04	0,46 ± 0,04	0,49 ± 0,02	0,49 ± 0,02	0,49 ± 0,02	0,46 ± 0,03	0,46 ± 0,03	0,48 ± 0,03	0,44 ± 0,02	0,49 ± 0,02	0,48 ± 0,02	0,44 ± 0,02	0,43 ± 0,02	0,47 ± 0,07	0,56 ± 0,04	0,70 ± 0,07	0,72 ± 0,06
ART WBC	4	281	0,45 ± 0,02	0,48 ± 0,04	0,46 ± 0,05	0,47 ± 0,04	0,48 ± 0,03	0,48 ± 0,07	0,50 ± 0,00	0,49 ± 0,02	0,50 ± 0,01	0,00 ± 0,00	0,50 ± 0,00	0,48 ± 0,02	0,47 ± 0,03	0,50 ± 0,00	0,49 ± 0,02	0,46 ± 0,03	0,46 ± 0,04	0,49 ± 0,02	0,48 ± 0,02	0,47 ± 0,03	0,47 ± 0,04	0,45 ± 0,06	0,47 ± 0,03	0,50 ± 0,03	0,57 ± 0,06	0,73 ± 0,04	0,75 ± 0,08

***rs10732516**

Appendix F. DNA methylation maps

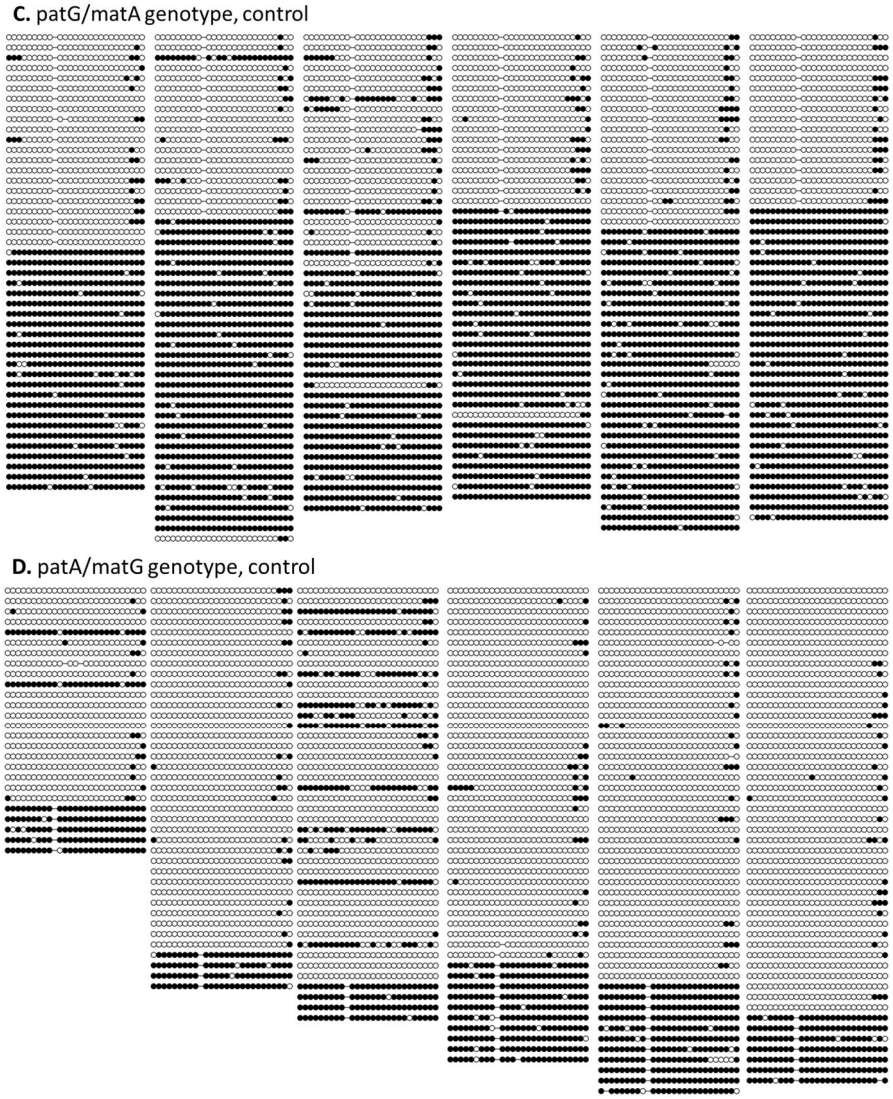

Figure 1. Visualization of DNA methylation profiles of 27 CpG sites at CTCF6 of *H19* ICR1 of ART derived placentas examined by bisulfite sequencing. A. Methylation profiles of ART samples with patG/matA **B.** Methylation profiles of ART samples with patA/matG genotype. **C.** Methylation profiles of control samples with patG/matA genotype. **D.** Methylation profiles of control samples with patA/matG genotype. Each circle represents individual CpG site. Black circles represent methylated CpG sites and white circles unmethylated CpG sites. Each line of CpG sites represents individual clone and one allele in one cell. Gaps represent A nucleotide at CpG10 site of rs10732516 G/A polymorphism. In ART derived placental samples, the paternal allele of patA/matG genotype was slightly hypomethylated compared to controls. Apparent PCR bias was detected in bisulfite sequencing: the amplification of hypomethylated maternal allele was favorited over hypermethylated paternal allele especially in patA/matG genotype.