BIRTH OUTCOMES, PLACENTAL METRICS AND IMPRINTED GENES IN PREGNANCIES CONCEIVED VIA ASSISTED REPRODUCTIVE TECHNOLOGIES

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Abstract

Infertility, the inability to achieve a natural pregnancy within twelve months, affects roughly 10% of couples worldwide. To address this difficulty, many people use assisted reproductive technologies (ART) such as *in vitro* fertilization, intracytoplasmic sperm injection, and intrauterine insemination. There is some concern over the safety of these techniques, however, as some adverse birth outcomes such as growth restriction, low fetoplacental weight ratio, and preterm birth, as well as certain developmental disorders known as imprinting disorders have been reported at higher rates in children conceived via ART. In this thesis, I investigate a number of birth outcomes and placental metrics as they relate to different factors of ART procedures. I also evaluate expression levels and DNA methylation (DNAme) profiles of imprinted genes in the context of births that display some of the adverse outcomes associated with ART. Birth weight, gestational age at birth, and APGAR scores at one and five minutes were not seen to be significantly different between ART and spontaneously conceived (SC) children. Placental metrics (weight, diameter, thickness), however, were seen to be significantly larger in ART children. These differences were most closely associated with which ART technique was used and whether or not it included *in vitro* embryo culturing. DNAme and expression profiles of imprinted genes associated with imprinting disorders did not differ significantly between births with low and normal fetoplacental weight ratios, despite previous studies finding significant differences between ART and SC in the same dataset. When only considering births that were preterm, growth-restricted, or of abnormal birth weight for their gestational age, expression of imprinted genes was not seen to be significantly different between ART and SC. The findings of these studies suggest that an increase in placental size is rather consistent among ART births, while the altered expression and DNAme profiles sometimes reported for ART are less common and more stochastic. There is also some evidence to suggest that the increased placental size is largely attributable to the *in vitro* culturing and ART techniques themselves, while the changes in DNAme and gene expression sometimes seen may be more attributable to the underlying infertility.

Lay Summary

In this thesis, I investigate a number of birth outcomes and placental metrics to compare births from natural pregnancies to those conceived using assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF). I found that although the newborns themselves were similar in both groups, the placentas of ART children were consistently larger, suggesting that ART techniques may be altering placental development. The lack of differences in genetic activity between ART and naturally conceived children in these studies would suggest that the genetic abnormalities sometimes seen in ART children are less consistent than the observation of larger placentas, and more likely due to random genetic abnormalities inherited from the infertile parents than the ART techniques.

Preface

This thesis was written with input and guidance from Dr. Wendy Robinson, Dr. Alex Beristain, and my supervisory committee members: Dr. Dan Rurak, Dr. Louis Lefebvre, and Dr. Wan Lam. These projects were designed by Samuel Schafer with significant input from Kenny Louie regarding the projects in Chapter 3. The experiments in Chapters 2 and 4 were performed by Samuel Schafer. The previous experiments that produced the data used in Chapter 3 were designed by Edgar Chan Wong and Rebecca Vincent and were performed by Rebecca Vincent and a former lab research assistant. Data analysis, tables, and figures were prepared by Samuel Schafer. Data collection was funded by the Canadian Institute of Health Research (grant number MOP-77549 to S.M.). A certificate of minimal risk approval was obtained from the University of British Columbia Children's and Women's Research Ethics Board (certificate number: H06-03668)

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List of Abbreviations

ART	assisted reproductive technologies
AS	Angelman syndrome
BWS	Beckwith-Wiedemann syndrome
CDKN1C	cyclin dependent kinase inhibitor 1C
cDNA	complementary DNA
CpG	cytosine-guanine dinucleotide
CTCF	CCCTC-binding factor
DMR	differentially methylated region
DNAme	DNA methylation, 5-mCpG
DNMT	DNA methyltransferase
FPR	fetoplacental weight ratio (birth weight / placental weight)
GIFT	gamete intrafallopian transfer
H19	*H19* imprinted maternally expressed transcript
HYMAI	hydatidiform mole associated and imprinted
ICR	imprinting control region
ICSI	intracytoplasmic sperm injection
IGF2	insulin like growth factor 2
IUGR	intrauterine growth restriction
IUI	intrauterine insemination
IVF	*in vitro* fertilization
KCNQ1	potassium voltage-gated channel subfamily Q member 1
KCNQ1OT1	*KCNQ1* opposite strand transcript 1
L1TD1	*LINE1* type transposase domain containing 1
LGA	large for gestational age (> 90^{th} percentile)
LINE1	long interspersed nuclear element 1
lncRNA	long non-coding RNA
MEG	maternally expressed gene
PCR	polymerase chain reaction
PEG	paternally expressed gene
PEG10	paternally expressed 10
PHLDA2	pleckstrin homology like domain family A member 2
PLAGL1	*PLAG1* like zinc finger 1
PWS	Prader-Willi syndrome
Rq	relative quantification
RT-qPCR	quantitative reverse transcription PCR
SAM	S-adenosylmethionine
SC	spontaneously conceived
SD	standard deviation
SGA	small for gestational age (< 10^{th} percentile)
SNRPN	small nuclear ribonucleoprotein polypeptide N
SRS	Silver-Russell syndrome
TNDM	transient neonatal diabetes mellitus

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Chapter 1: Introduction

1.1 Project overview

As of 2018, over 8 million children had been born to assisted reproductive technology (ART) since its introduction four decades ago [1]. That number continues to increase as approximately 2.4 million ART cycles are now being performed each year, resulting in about 500,000 births worldwide annually [1]. Despite an increased risk for some adverse perinatal outcomes, the vast majority of ART births are without serious complications and result in normal, healthy children [2-5]. However, a well-known and widely accepted hypothesis in developmental biology has raised concerns over the long-term effects of ART. This idea, known as the developmental origins of health and disease (DOHaD) hypothesis, proposes that environmental conditions experienced around the time of gamete maturation, fertilization and throughout gestation may result in adaptive responses by the fetus that can persist into postnatal life and continue to affect an individual's health into adulthood. This hypothesis, largely inspired by David Barker's studies in the late 1980s and early 1990s [6-9], has given researchers reason to more closely investigate the increased risk of adverse birth outcomes in ART, despite the absolute number of affected pregnancies still being low. By conducting a retrospective analysis of over a decade of birth data, this thesis seeks to shed light on which elements of the ART process may be contributing to the increased risks that have been reported. Gene expression and DNA methylation (DNAme) patterns of imprinted genes are also evaluated amongst newborns that display adverse or atypical birth outcomes to determine if the observed effects are associated with abnormal expression of genes that regulate embryonic growth and placental function.

1.2 Infertility

Infertility is most commonly defined as the inability to achieve pregnancy after one year of intercourse without the use of contraceptives. The causes of infertility affect both men and women and are extremely variable, including such wide-ranging things as advanced age [10], genetic abnormalities [11], exposure to environmental toxins or radiation [12,13], tissue damage resulting from disease [14,15], and physical trauma [16]. Considering all forms of infertility, it is

estimated to affect approximately 10% of couples worldwide [17], and for the last several decades, there have been claims that this rate is increasing [18,19]. There is much doubt, however, as to whether this is a genuine increase in the prevalence of infertility. It has been suggested that it is instead an increase in the reporting of infertility, a biased perspective due to fertility data increasingly being provided by clinics that serve patients experiencing fertility issues, or some combination of the two. Regardless of whether or not the prevalence of infertility is rising, the number of people seeking infertility treatments like ART is certainly increasing [1].

1.3 Assisted reproductive technologies

1.3.1 Overview of ART techniques

The term ART most often refers to any clinical technique that employs *ex vivo* handling of both egg and sperm cells. By this definition, only *in vitro* fertilization (IVF), variations of IVF such as intracytoplasmic sperm injection (ICSI), and the far less common gamete intrafallopian transfer (GIFT), are considered ART. For the purpose of considering infertile couples who did not use *in vitro* culturing of the oocyte or embryo, intrauterine insemination (IUI) is also considered an ART throughout this thesis. The process of IUI involves collecting a sperm sample from either the male partner or a donor and using a catheter to inject the sample directly into the patient's uterus, typically in the upper portion near the fallopian tubes to better facilitate fertilization. Because this technique does not involve external handling of the oocyte, it is very common in cases with a fertile female and the use of a sperm donor. It also offers the benefit of significantly reducing the distance that spermatozoa must travel to reach the oocyte, making it an effective treatment for cases of mild to moderate low sperm count or motility [20].

For infertile couples that are unable to achieve pregnancy with the more affordable and less invasive IUI procedure, the most common solution is IVF. It involves the collection of a sperm sample as in IUI but also requires the retrieval of mature oocytes from the ovary using an ultrasound-guided needle. This is done about 36 hours after a hormonal injection (either human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH) agonist, or both) is administered to induce the final stage of oocyte maturation [21]. The egg and sperm samples are then incubated together in a culture medium to allow fertilization to take place. When a second pronucleus is visible in the oocyte, indicating a successful fertilization, the cell is transferred to

an embryonic growth medium where it is cultured for two to six days, until the embryo has reached either the cleavage or blastocyst stage, depending on the specific clinic's protocol [22,23]. At this point, the embryo is transferred to either the mother or a gestational surrogate's uterus or frozen for transfer at a later date. Because IVF cycles often result in multiple embryos, those that are not used are generally frozen for future cycles or donation to research or other infertile couples. More than one embryo can be transferred in a single cycle to increase the odds of implantation, but due to the relatively high success rates that are currently seen and the additional complications associated with multiple gestation, many countries limit transfers to just two embryos per cycle except in special circumstances [24,25]. ICSI, the most common form of IVF, is often recommended in cases of severe male infertility. Because it involves directly injecting a single spermatozoon into the cytoplasm of an oocyte using a micropipette, it can be performed in cases where sperm count, motility, and morphology are extremely poor.

1.3.2 Results and risks of ART

Though success rates vary significantly from one clinic to another, ranging from 10-40% for individual clinics, the global average for the rate of live births per ART cycle is generally reported to be about 20-25% [26]. Of the approximately 500,000 live births that occur each year as a result of ART, the majority do not experience complications and produce normal, healthy children [2-5]. The rates of some complications, however, are somewhat higher than they are in spontaneously conceived (SC) births. Preterm parturition, for instance, has been reported to have a relative risk (RR) of about 1.5 to 1.8 in ART singleton births compared to spontaneously conceived singleton births [27,28]. Low birth weight also appears to be significantly more common in ART singleton births (RR 1.6) [28]. Twin births also showed an increased risk, though to a lesser degree (RR 1.1 - 1.2) [29]. In addition to pregnancy outcomes such as gestational age and weight at birth, some metrics of the placenta have also been reported to be altered. Several studies have suggested larger or heavier placentas in ART pregnancies [30-34]. At least one study in mice also found a higher placental weight in ART pregnancies, noting the greatest difference was seen in late pregnancy when the ART placentas continued growing after spontaneously conceived placenta growth had plateaued [33]. Though there is some debate as to whether it is attributable to a decreased birth weight, an increased placental weight, or both, a metric known as the fetoplacental weight ratio (FPR) has been quite consistently reported to be

decreased in ART births [30,31,34]. This metric is calculated by dividing an infant's birthweight in grams by its placental weight in grams. An increased frequency of rare developmental disorders called imprinting disorders has also been reported numerous times, though their rarity makes it difficult to accurately assess the extent of the difference in frequency [35-39].

1.4 Imprinted genes

1.4.1 Mechanisms

The use of ART to achieve pregnancy has been associated with an apparent increase in some imprinting disorders, developmental disorders in which the regulation of imprinted genes is disrupted. Imprinted genes are genes for which only one allele is expressed depending on the parent from which it was inherited. This mono-allelic expression of imprinted genes is regulated by epigenetic mechanisms that can affect gene expression without altering the genetic sequence. The most studied and best understood epigenetic mechanism, particularly in the context of imprinting, is DNAme. This is a molecular process in which enzymes known as DNA methyltransferase (DNMT) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the carbon atom in position 5 of a cytosine nucleotide (Figure 1.1). In mammals, this occurs almost exclusively at CpG dinucleotides, which are far less prevalent than would be expected based solely on the genome's composition of guanine and cytosine nucleotides [40,41]. However, some genetic regions, known as CpG islands, are rich in C+G nucleotide pairs and are most often located within gene promoters [41,42]. Many CpG island promoters are unmethylated whether the gene is expressed or not, but when high-density DNAme is present within a gene promoter, it is usually associated with the repression of transcription [41,43]. This is understood to be the effect of the methyl groups' presence physically blocking transcription factors from binding to the promoter region, and recruiting repressive methyl-binding proteins [43].

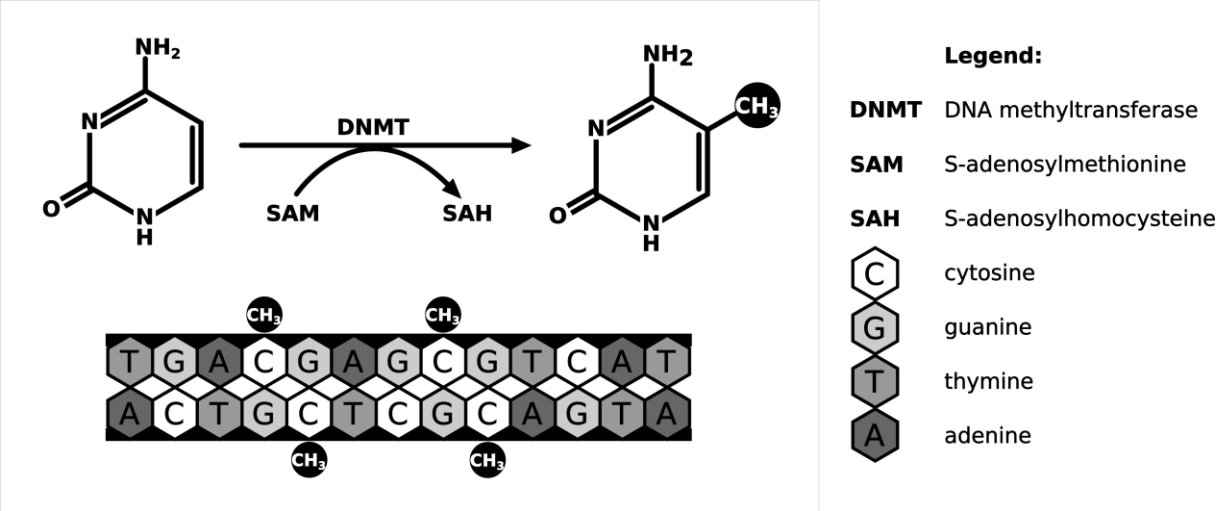


Figure 1.1. Mechanism of DNAme placement. Diagram depicting placement of DNAme marks on CpG dinucleotides by DNMT.

The ability of DNAme to restrict gene expression is known to play a key role in the allele-specific silencing of at least some imprinted genes, though other epigenetic elements, such as histone tail modifications and in *cis* silencing by long non-coding RNAs (lncRNA), have also been implicated in the mechanism of imprinting, either as direct regulators or consequences of CpG-rich regions being methylated [43,44]. Even within the mechanism of DNAme alone, there are primary imprints that regulate the placement and maintenance of secondary imprints. This is best demonstrated by the existence of imprinting control regions (ICRs), genomic elements that, depending on their DNAme status, will differentially induce hyper- or hypomethylation at nearby gene promoters that in turn, can directly affect transcription levels of the target genes bearing the DNAme marks[45]. The 11p15.5 locus, shown in Figure 1.2, is one of the most well-studied examples of an imprinted gene cluster in the human genome and illustrates this imprinting mechanism well. It contains two differentially methylated regions (DMRs) that together regulate the expression of at least ten different genes, including the maternally-expressed *PHLDA2*, *CDKN1C*, and *H19*, as well as the paternally-expressed *KCNQ1OT1* and *IGF2* [46,47]. One of these differentially-methylated regions, DMR1, is located between *H19* and *IGF2* and regulates their parental allele-specific expression pattern. When DMR1 is unmethylated, as normally seen on the maternal allele, the zinc-finger protein, CCCTC-binding

factor (CTCF), binds to DMR1. This establishes an insulator that blocks access to *IGF2* for the downstream enhancer shared by *IGF2* and *H19*, resulting in only *H19* being expressed from the allele. When DMR1 is methylated however, as normally seen on the paternal allele, CTCF does not bind and the enhancer is allowed access to the *IGF2* promoter, which will be expressed preferentially instead of *H19* [48]. The other ICR in this region, DMR2, exemplifies a different method of gene regulation as it is the promoter of the lncRNA, *KCNQ1OT1*. When DMR2 is methylated, as normally seen on the maternal allele, transcription factors are unable to access the *KCNQ1OT1* promoter and the lncRNA is not expressed. When DMR2 is unmethylated however, as normally seen on the paternal allele, *KCNQ1OT1*'s promoter is accessible and the gene is expressed. The resulting lncRNA, which is transcribed antisense to the *KCNQ1* gene from one of its introns, interacts with the chromatin in *cis* and represses the transcription of multiple nearby genes through a molecular mechanism that is less clearly understood but likely involves the recruitment of other epigenetic components leading to histone tail modifications [47].

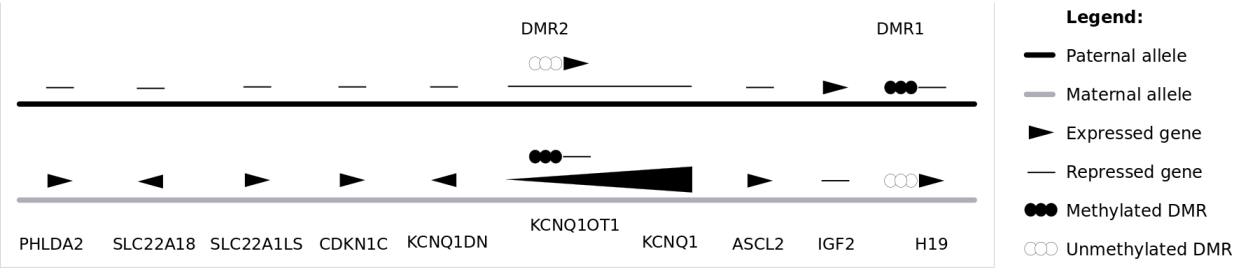


Figure 1.2. Imprinting control regions at 11p15.5. Diagram depicting differential DNAme and expression of maternal and paternal alleles at 11p15.5.

1.4.2 Role in development

Most imprinted genes have been suggested to be a manifestation of intragenomic sexual conflict in which the male epigenome and female epigenome are optimized for differing and competing reproductive and developmental strategies [49]. This is supported by studies that have found that paternally-expressed imprinted genes (PEGs) often increase the effectiveness of trophoblast invasion into the endometrium, placental growth and efficiency, fetal growth, and longer gestation. Maternally-expressed imprinted genes (MEGs) however, often reduce the effectiveness of those processes, preserving maternal reproductive fitness by reducing the risk of

fetal overgrowth and the damage it can cause to the mother's subsequent health and fertility [50]. Given this role of regulating placentation and fetal development, it is not surprising that there are many imprinted genes and splice variants of imprinted genes that are unique to the placenta [51,52]. Additionally, biallelic expression of certain imprinted genes is more common in the placenta than in somatic tissues, often with no apparent disorder or phenotype associated [52,53].

1.4.3 Imprinting disorders

Though there are exceptions in placental tissues, anything other than one active copy of each imprinted gene will often result in a failed or abnormal embryo. It is still possible for an embryo to develop to term with a very small number of abnormally expressed imprinted genes, but such cases usually result in severe post-natal developmental disorders known as imprinting disorders (Table 1.2). These arise when one of the following errors occurs in an imprinted gene; deletion, duplication, point mutation, uniparental disomy, aneuploidy, or epigenetic abnormalities (e.g., hypomethylation or hypermethylation at ICRs) [54]. The effect that these issues have in common, and what leads to the clinical symptoms of imprinting disorders, is the active transcription of any number other than exactly one copy of the imprinted gene. If a MEG displays biallelic expression or a PEG displays no expression, an undergrowth phenotype is often seen, as in Silver-Russell syndrome (SRS) [55]. If the inverse is true, biallelic expression of a PEG or no expression of a MEG, an overgrowth phenotype is often observed, as in Beckwith-Wiedemann syndrome (BWS) [55]. While this is true of some imprinting disorders and reflects the intragenomic sexual conflict aspect of many imprinted genes, other imprinted genes are less involved in overall growth as they are in the development of a particular system. Angelman syndrome (AS) and Prader-Willi syndrome (PWS), for example, are caused by inverse errors at a single genetic region, much like BWS and SRS [55]. Rather than resulting in overgrowth and undergrowth phenotypes, however, they both produce abnormal neural development and mental deficiency along with some seemingly unrelated symptoms throughout the body such as hypopigmentation and abnormalities in speech and feeding [55]. This is because the affected gene, *SNRPN*, is most highly expressed in the brain, spinal cord and heart, and its involvement in tissue-specific alternative RNA splicing is critical for neural development [56]. Transient neonatal diabetes mellitus (TNDM), another imprinting disorder, is unlike BWS, SRS, PWS, and AS in that it has no known "sister" disorder associated with the same genetic locus, 6q24 [57].

Tables 1.1 and 1.2 briefly describe some relevant imprinting disorders and the imprinted genes associated with them [55,57,58].

Disorder	Common Clinical Traits	Associated Genes
SRS	Small stature, skeletal asymmetry (limbs), characteristic facial appearance, clinodactyly	*IGF2, H19, CDKN1C*
BWS	Macrosomia, characteristic facial appearance, macroglossia, omphalocele, hypoglycemia, organomegaly, abdominal tumors, hemihypertrophy	*PHLDA2, IGF2, KCNQ1OT1, H19, KCNQ1, CDKN1C*
AS	Severe mental deficiency, motor dysfunction, speech impairment, frequent seizures, hypopigmentation, hyperactivity, characteristic facial appearance	*SNRPN*
PWS	Hypotonia, poor feeding in infancy, characteristic facial appearance, hyperphagia, behavioral problems hypogonadism/hypogenitalism, small stature, small hands/feet, hypopigmentation, mental deficiency	*SNRPN*
TNDM	Macroglossia, umbilical hernia, characteristic facial appearance, renal tract abnormalities, cardiac anomalies, clinodactyly, polydactyly, hypothyroidism	*HYMAI, PLAGL1*

Table 1.1. Imprinting disorders and associated genes. Brief descriptions of clinical traits of five relevant imprinting disorders and imprinted genes associated with them.

Gene	Functional Role	Expressed Allele
IGF2	Insulin family polypeptide growth factor	Paternal
H19	Tumor suppressing lncRNA	Maternal
CDKN1C	Cell growth suppressing protein	Maternal
PHLDA2	Placental growth suppressing protein	Maternal
KCNQ1OT1	Transcription regulating lncRNA	Paternal
KCNQ1	Voltage-gated potassium channel protein	Maternal
SNRPN	RNA alternative splicing regulating protein	Paternal
HYMAI	ncRNA (function unclear)	Paternal
PLAGL1	Cell growth suppressing zinc-finger protein	Paternal

Table 1.2. Roles of genes associated with imprinting disorders. Brief descriptions of biological roles and expressed parental alleles of imprinted genes studied in this thesis.

1.4.4 Effects of ART on imprinted genes

Although imprinting disorders are significantly more prevalent in ART children, their absolute risk is still very low [35-39]. In the general population, imprinting disorders only occur in one out of every 10,000 or more births. Some only occur once in every 100,000 - 400,000

births, and for others the frequency is still unknown because of how rarely they are observed and reported [59]. Even with the increased risk that has been reported for ART births, one meta-analysis found SRS, BWS, AS, and PWS, to have odds ratios of 11.3, 5.8, 4.7, and 2.2 respectively, for ART births [35], the prevalence is still less than half of a percent [60].

Despite the absolute risk being quite low, the higher relative risk has caused researchers and clinicians alike to question whether or not the ART procedures are affecting imprinted genes, especially since they are performed during a period of gametogenic and embryogenic processes where imprinting maintenance is critical. This period contains two major DNAme erasure and replacement events, the first of which occurs in the primordial germ cells of the parents undergoing ART. Aside from retrotransposons, most of which remain at least partially methylated to inhibit their transposable mobility, this first event erases all DNAme marks throughout the genome, including imprinted DMRs. This provides the primordial germ cells with a blank slate epigenome, devoid of any activating or repressive epigenetic marks, so that the sex-specific marks inherited from the individual's parents can be replaced with new ones according to their own sex [61]. In males, this begins prenatally and is completed by the pachytene phase of postnatal spermatogenesis, before even the earliest surgical sperm retrieval techniques used in cases of extreme oligospermia. Oocytes, however, do not begin placing maternal marks until after birth. The majority of the DNAme is placed during postnatal oocyte growth and follicular development, with the final marks being placed around the time of ovulation, just before fertilization occurs [62,63]. Because this is the period during which hormonal stimulation is used to artificially increase the number of oocytes being matured and released in most ART procedures, it is not surprising that several studies have reported abnormal maternal DNAme patterns associated with hormonal superovulation in both humans and animal models [64-66]. These abnormalities in DNAme can, in turn, alter expression levels of imprinted genes.

The second major DNAme erasure event takes place in the preimplantation phase, between the zygotic and blastocyst stages of the newly formed embryo. Imprinted genes retain their epigenetic marks through this erasure event to ensure they are expressed at the proper levels later during the postimplantation phase, since one of their primary functions is to regulate embryonic development. The majority of the genome, however, is cleared of DNAme so that tissue-specific and environmentally responsive epigenetic marks can be replaced in the context

of the newly formed embryo and its environment, rather than preserving the epigenomes of its parents' gametes [67,68]. In ART, this second erasure event takes place entirely during the preimplantation period in which the embryo is being cultured *in vitro*, causing some concern that the artificial environment may be altering the way DNAme marks are placed, erased, or maintained given their responsiveness to environmental factors [69].

The idea that ART procedures may be disrupting the establishment of normal DNAme patterns would suggest that the increased rate of imprinting disorders seen in ART is due to hypo- or hypermethylation as opposed to deletions, duplications, point mutations aneuploidy, or uniparental disomy. There is some evidence of this as at least one study has shown altered DNAme to be the cause of fewer than 60% of BWS cases in the general population but over 95% of cases within the ART population [70]. The same study also found altered DNAme to be responsible for fewer than 5% of AS cases in the general population, but about 25% of cases in the ART population. This seems to strongly suggest that ART is affecting DNAme patterns in cases of imprinting disorders, but when DNAme is studied directly, the picture is far less clear. Although several studies have reported differences in the DNAme levels of ART and SC children, the findings lack consistency. Some studies find hypermethylation, others find hypomethylation, and others still see no significant differences, all within the same genetic regions [71,72]. Of those studies that did observe significant differences, the majority of them did not find a difference that exceeded the range of natural variation of DNAme levels seen in the general population. Some significant studies of DNAme and imprinted gene expression in ART have been summarized below in Table 1.3 [73-84]. Considering the consistently observed increase in imprinting disorders and the more stochastic observations of DNAme changes, it may be that some ART pregnancies are prone to DNAme errors, but not necessarily as a result of the procedures themselves, otherwise, a more consistent effect would be expected.

First Author	Year	Tissue	Sample Size	# Genes	Main Result
Choux	2018	Cord blood Placenta	ICSI = 36 IVF = 15 SC = 48	7	↓ *ERVFRD-1* expr (cb, pl) ↓ *LINE1* expr (cb) ↓ *H19/IGF2* DNAme (pl) ↓ *KCNQ1OT1* DNAme (pl) ↓ *LINE1*Hs DNAme (pl) ↓ *ERVFRD-1* DNAme (pl)
Gentilini	2018	Cord blood	ART = 23 SC = 41	450K (CpGs)	No sig diff
Ghosh	2017	Placenta	LUMA: ICSI = 54 IVF = 127 SC = 77 Pyrosequencing: ICSI = 39 IVF = 87 SC = 65	LUMA: >2.3 million (CCGGs) Pyrosequencing: 1	↑ global DNAme ↓ *LINE1* DNAme (fresh transfers only)
Katagiri	2010	Placenta	ART = 65 SC = 924	4	No sig diff in expr (AGA) ↓ *H19* expr (SGA; ART, SC) ↓ *CDKN1C* expr (SGA; ART) ↑ *KCNQ1OT1* expr (SGA; SC)
Katari	2009	Cord blood Placenta	8 genes: IVF = 60-73 SC = 63-100 3 genes: IVF = 22-25 SC = 29-34	700 (1536 CpGs)	↓ 13 CpGs DNAme (cb) ↑ 12 CpGs DNAme (cb) ↓ 11 CpGs DNAme (pl) ↑ 3 CpGs DNAme (pl)
Li	2011	Cord blood	IVF/ICSI = 29 SC = 30 (# of twin pairs)	3	No sig diff in DNAme
Litzky	2017	Placenta	IVF = 18 subfertile = 79 SC = 158	108	45 genes expr sig diff (subfertile) 0 genes expr sig diff (IVF)
Nelissen	2013	Placenta	ICSI = 30 IVF = 5 SC = 35	10 (DMRs)	↓ *MEST* DNAme ↓ *H19* DNAme ↑ *H19* expr
Nelissen	2014	Placenta	ART = 81 SC = 105	6	↑ *H19* expr ↑ *PHLDA2* expr Changes not due to LOI
Rancourt	2012	Cord blood Placenta	IVF = 59 OI = 27 SC = 61	6 (DMRs)	↓ *H19* DNAme (OI pl) ↑ *KCNQ1* DNAme (OI cb) ↑ *SNRPN* DNAme (OI cb, pl) ↓ *H19* DNAme (IVF pl) ↓ *MEST* DNAme (IVF pl) ↑ *SNRPN* DNAme (IVF pl) ↑ *KCNQ1* DNAme (IVF cb) Only *H19* was functionally relevant
Song	2015	Placenta	ART = 66 DO = 22 SC = 49	37 (CpGs)	11 CpGs DNAme sig diff (between all groups) 7/11 CpGs DNAme sig diff (donor)
Zheng	2011	Cord blood	IVF = 61 ICSI = 40 SC = 60	6 (DMRs)	No sig diff in DNAme

Table 1.3. Studies of DNAme and gene expression in ART. Brief summaries of twelve studies evaluating DNAme and gene expression in umbilical cord blood and placental tissue from ART births. expr, gene expression; cb, cord blood; pl, placenta; OI, induced ovulation; DO, donor oocyte.

1.5 Rationale

1.5.1 Hypotheses and objectives

In this thesis, I hypothesize that either some element of ART techniques or the infertility that necessitates their use is associated with atypical birth outcomes and placental metrics. To test this hypothesis, a statistical analysis will be performed using a database of pregnancy and birth information, grouping the cases by a number of different factors of the ART process. It is hoped that this analysis will not only reconfirm the altered birth outcomes reported for ART pregnancies but also shed some light onto which factors are most responsible for those outcomes. I hypothesize that the lower FPR consistently observed in ART births is associated with alterations in the expression levels or DNAme patterns of imprinted genes in ART births. To address this, gene expression and DNAme data that was collected in previous studies of imprinted genes will be reanalyzed with the goal of comparing births with low FPRs to those with average ratios, regardless of ART status. This evaluation will address the matter of whether or not a low weight ratio is indicative of an altered genetic or epigenetic state. Additionally, I hypothesize that among births displaying adverse outcomes such as low birth weight and preterm parturition, ART births will stand out as having a distinct pattern of altered expression in imprinted genes. To test this, expression levels of four imprinted genes, *H19*, *HYMAI*, *PLAGL1*, and *PHLDA2*, will be evaluated in the umbilical cord blood of pregnancies that resulted in preterm birth, SGA birth weight, LGA birth weight, or IUGR. It is anticipated that this study will provide insight into whether the adverse outcomes seen in ART are random or due to a distinct effect of the clinical procedures.

Objective 1a: To compare birth outcomes and placental metrics of ART and SC pregnancies.

Objective 1b: To compare the relative impact of different components of ART on birth outcomes and placental metrics.

Objective 2: To compare expression levels and DNAme patterns of imprinted genes in ART and SC pregnancies.

Objective 3: To compare expression levels of imprinted genes in ART and SC pregnancies with adverse birth outcomes.

Chapter 2: Birth outcomes and placental metrics in ART and SC pregnancies

2.1 Introduction

As the outcome and safety of ART pregnancies have been the focus of numerous studies in the past few decades, a number of publications have reported differences in both birth outcomes and placental metrics between children of ART and SC pregnancies [2-5]. Gestational age at birth, birth weight and size, placental weight and size, and FPR are among the most commonly reported, with ART pregnancies typically displaying earlier births [3,4,27,28], smaller birth size [3,4,28], larger placental size [30-34], and lower FPRs [30,31,34]. The question remains, however, are these changes due to the ART techniques themselves or the subfertility that necessitates their use? It is possible that couples who struggle with fertility are unable to conceive without assistance because their gametes are in some way suboptimal (e.g., containing epigenetic errors), leading to delayed or otherwise compromised development. Alternatively, the ART techniques (e.g., cell membrane puncture in ICSI) or their effects (e.g., additional psychological stress) may be disrupting normal embryogenesis and resulting in an altered developmental trajectory.

Because so many births today occur in hospitals where numerous assessments are made at birth in a manner that is consistent throughout the industrialized world, these measurements can be used to more precisely understand the observed differences between ART and SC births. When taken together, birth weight, body length, and head circumference form a clear picture of a newborn's size at birth, especially when corrected for gestational age. The Apgar score was invented in 1952 by an anesthesiologist, Dr. Virginia Apgar, to quickly assess the general health and vitality of a newborn at the one-minute and five-minute marks after delivery [85]. It is the sum of five scores, ranging from 0-2, in the areas of skin color, heart rate, reflex irritability, muscle tone, and respiration. In addition to the standard hospital assessments, the placenta can be collected and measured for weight, diameter, thickness, and umbilical cord diameter, which can offer some insight into its functional efficiency.

Of all the metrics listed above, gestational age, birth weight, and placental weight are most consistently reported to be different between ART and SC births [3,4,27-31], so it is

expected that they will be seen to be altered in this analysis as well. Because ART newborns are expected to display lower gestational age and birth weight but higher placental weight, it logically follows that FPR is also expected to be lower in the ART group, especially since it has been observed that FPR tends to increase throughout the latter half of pregnancy [86]. If any one element of treating infertility, such as the infertility itself or the *in vitro* culturing of the embryo, is primarily responsible for driving the differences in birth outcome, it should display similar or even greater differences in gestational age, birth weight, placental weight, and FPR.

In addition to the common ART versus SC comparison, and the breakdown of each specific conception mode on its own, seven other divisions were used to evaluate the source of the differences between ART and SC newborns. Because the *in vitro* culturing of embryos in IVF and ICSI has often been pointed to as one potential cause of alterations in ART [87-89], it has been included as a comparison point. An analysis grouped by whether or not an embryo was cultured *in vitro* should have similar results to the ART versus SC comparison, with any differences being attributable to the IUI group that in this analysis is considered an ART but does not involve embryo culturing. Another element of ART that has been reported to affect birth outcomes [90-94], and was therefore used as a factor for comparison, is the freezing of embryos prior to transfer for implantation. Several studies have suggested that transferring frozen embryos has a lower risk of preterm birth and low birth weight than fresh ART cycles [91-94], though whether this is due to the further separation of hormonal superovulation and implantation dates or the freezing process itself is unclear. As it is yet another element that has been hypothesized to contribute to the alterations seen in ART, infertility was also considered. Samples were divided into groups that showed infertility in the male partner, female partner, both, or neither to consider whether the source of infertility would impact the results, as well as to determine if those cases where ART was used in the absence of infertility would align more closely with the ART or SC cases. To determine if the use of gamete donors or a surrogate contributed additional risk on top of the ART alone, they were also included as factors for comparison. Lastly, the mother's gravidity, or total number of pregnancies, was also included, primarily as a reference point against which the other comparisons could be evaluated. Gravidity was selected for its effect of increased birth size in successive pregnancies, which is somewhat

commonly known and seems intuitive even to lay people, but is still weak enough to be comparable to the minor differences observed in ART studies.

2.2 Methods

2.2.1 Study participants

A total of 792 pregnancies resulting in 889 live births were recruited primarily in the greater Vancouver, BC area, though a small percentage were recruited from other cities across Canada. Both ART and SC participants were identified through their fertility clinics, physicians, and hospitals after expressing interest in research participation. They then gave written informed consent for the collection of placenta and umbilical cord blood samples after parturition (ethics certificate number: H06-03668). No exclusion criteria were applied in the recruitment of participants. The cohort consisted of 330 pregnancies (340 newborns) that were conceived spontaneously within one year of trying and 462 (549) that were conceived via ART. Of the ART pregnancies, 194 (235) were conceived via IVF, 228 (268) via ICSI, 27 (29) via IUI, and an additional 13 (17) were conceived via *in vitro* fertilization though it was unclear whether the fetus resulted from a traditional IVF or ICSI embryo. Of the 889 newborns recorded in the database, 700 were singletons, 174 were twins (87 pairs), and the remaining 15 were triplets (5 sets)(Table 2.1). In four of the twin pregnancies, data was only received for one of the twins, meaning there was only data available for 170 of the 174 newborns in the analysis of the twin database.

	SC	IUI	IVF	IVF/ICSI	ICSI	**Total**
Singletons	320	25	155	9	191	**700**
Twins	20	4	74	8	68	**174**
Triplets	0	0	6	0	9	**15**
Total	**340**	**29**	**235**	**17**	**268**	**889**

Table 2.1. Newborns in database by mode of conception. Table summarizing number of newborns born as singletons, twins, or triplets per conception mode. SC, spontaneous conception; IUI, intrauterine insemination; IVF, *in vitro* fertilization; IVF/ICSI, *in vitro* fertilization or intracytoplasmic sperm injection; ICSI, intracytoplasmic sperm injection.

2.2.2 Data and sample collection

Participants were provided with a questionnaire for self-reporting relevant clinical information regarding their current and previous pregnancies. Over time, this questionnaire was expanded to include more information as research studies revealed new points of interest. Therefore, later pregnancy cases generally had more data available than the earlier cases. A birth outcome form was also provided to delivery room staff for collecting data such as birth weight and gestational age at birth.

Within 30 minutes of the birth, delivery room staff drew approximately 10mL of blood from the placental umbilical cord and biopsied three $1cm^3$ samples of chorionic villous tissue from the fetal side of the placenta of each newborn. The three villous tissue samples were consistently taken from sites located adjacent to the umbilical cord insertion site, near the edge of the chorionic plate, and at the midpoint of the first two sites. The villous tissue samples were placed in 3mL of RNAlater (Sigma-Aldrich, St. Louis, MO) and, along with the umbilical cord blood sample and whole placenta, were stored at 4°C for up to 48 hours until further processing.

2.2.3 Sample processing

Within 48 hours of the birth, the placenta was processed, measured, and biopsied. The placenta processing consisted of removing the amniotic sac, draining the placenta of blood, weighing the drained placenta, and measuring the diameter of the umbilical cord, the maximum diameter of the placenta (labeled as x-axis), the diameter of the placenta perpendicular to the maximum diameter (labeled as y-axis), and the maximum and minimum thickness of the placenta. Ten additional $1cm^3$ chorionic villous tissue samples were biopsied for DNA extraction after all measurements were taken. These biopsies were taken from the same locations as the first three with seven additional random sites around the placenta. All thirteen villous tissue samples were snap-frozen on dry ice and stored at -80°C.

2.2.4 Database analysis

The database is an accumulation of birth outcome and placental metric data from the past 16 years of patient recruitment for infertility and pregnancy outcome studies. The data was analyzed by comparing outcomes across different modes of conception as well as several other factors that have been proposed to play a role in creating the differences seen between ART and

naturally conceived pregnancies. In addition to the specific mode of conception, the use of any ART, use of embryo culture, mother's gravidity, source of infertility, type of embryo transfer, use of a sperm donor, use of an egg donor, and use of a surrogate were each used as factors in comparing outcomes. The birth outcomes and placental metrics evaluated as variables were the gestational age at birth, birth weight, newborn body length, head circumference, Apgar score at one minute, Apgar score at five minutes, weight of the trimmed and drained placenta, FPR, diameter of the placenta on the x-axis, diameter of the placenta on the y-axis, y diameter to x diameter ratio, minimum and maximum thickness of the placenta, and diameter of the umbilical cord. Follow-up comparisons of the variables showing the most significant differences were performed using ART status and either the source of infertility or the use of embryo culturing as factors. Only singleton data was used in these follow-up comparisons as the twin data had insufficient sample sizes for calculation. Because birth weight, length, and head circumference are known to be correlated with gestational age [95-97] and gestational age was expected to vary significantly between conception modes, the Fenton growth chart [98] was used to produce a percentile value that could more accurately be compared across groups of variable gestational age. Data for singletons and twins were analyzed and reported separately.

2.2.5 Statistical analysis

All statistical analyses were performed in the statistical program R (3.5.2) and its user interface software RStudio (1.1.456). In testing for associations in this database analysis, the Mann-Whitney U test or Kruskal-Wallis H test was performed for comparisons of two factors or more than two factors, respectively, for all categorical data and any numerical data that did not display a normal distribution. For all normally distributed numerical data, test statistics were determined using the Tukey HSD test or ANOVA test, depending on whether the comparisons were between two factors or more. Each Kruskal-Wallis H test and ANOVA test that showed a significant difference ($\alpha = .050$) was followed up with pairwise comparisons using the Mann-Whitney U test or Tukey HSD test, respectively. Although a minimum confidence interval of 95% ($\alpha = .050$) was used throughout this database analysis for reporting significance, the Bonferroni corrections of $\alpha = .050/17 = .0029$ for seventeen variables and $\alpha = .050/153 = .0003$ for seventeen variables across the nine different groupings are used throughout this chapter to keep the high degree of multiple testing in perspective. For this reason, *p* values for the birth

outcome analyses in sections 2.3 and 2.4 were reported to four decimal places instead of the conventional three and asterisks are used to denote *p* values equal to or less than .0500 (*), .0029 (**), and .0003 (***) instead of the conventional .050 (*), .010 (**), and .001 (***). Analysis of the clinical information was done using the conventional $\alpha = .050$ without correction.

2.3 Results (singletons)

2.3.1 Clinical information

As expected, pregnancies in each ART group had a higher maternal age when compared to the SC group: IVF ($p < .001$), ICSI ($p < .001$), IVF/ICSI ($p = .001$), IUI ($p = .027$). There were also fewer IUGR births in the IVF ($p = .003$) and ICSI ($p < .001$) groups when compared to the SC group (Table 2.2). Percentages reported for female, IUGR, LGA, and SGA births were calculated using the cases in each group for which the data was known, not necessarily all the cases in each group.

	SC	IUI	IVF	IVF/ICSI	ICSI	*p* value
n (*N* = 700)	320	25	155	9	191	
Mean Mat. Age (years ± *SD*)	32 ± 5 ^{a}	35 ± 4 ^{b}	36 ± 4 ^{b}	38 ± 4 ^{b}	35 ± 4 ^{b}	< .001 ***
Mean Mat. BMI (± *SD*)	25 ± 6	25 ± 5	25 ± 6	24 ± 4	25 ± 5	.984
Female (%)	51 (154/300)	48 (11/23)	52 (62/119)	25 (2/8)	42 (47/111)	.296
IUGR (%)	10 ^{a} (33/320)	0 ^{ab} (0/25)	3 ^{b} (4/155)	0 ^{ab} (0/9)	2 ^{b} (4/191)	< .001 ***
LGA (%)	8 (24/291)	22 (5/23)	9 (9/99)	0 (0/8)	3 (3/86)	.060
SGA (%)	11 (33/291)	13 (3/23)	6 (6/99)	0 (0/8)	8 (7/86)	.436

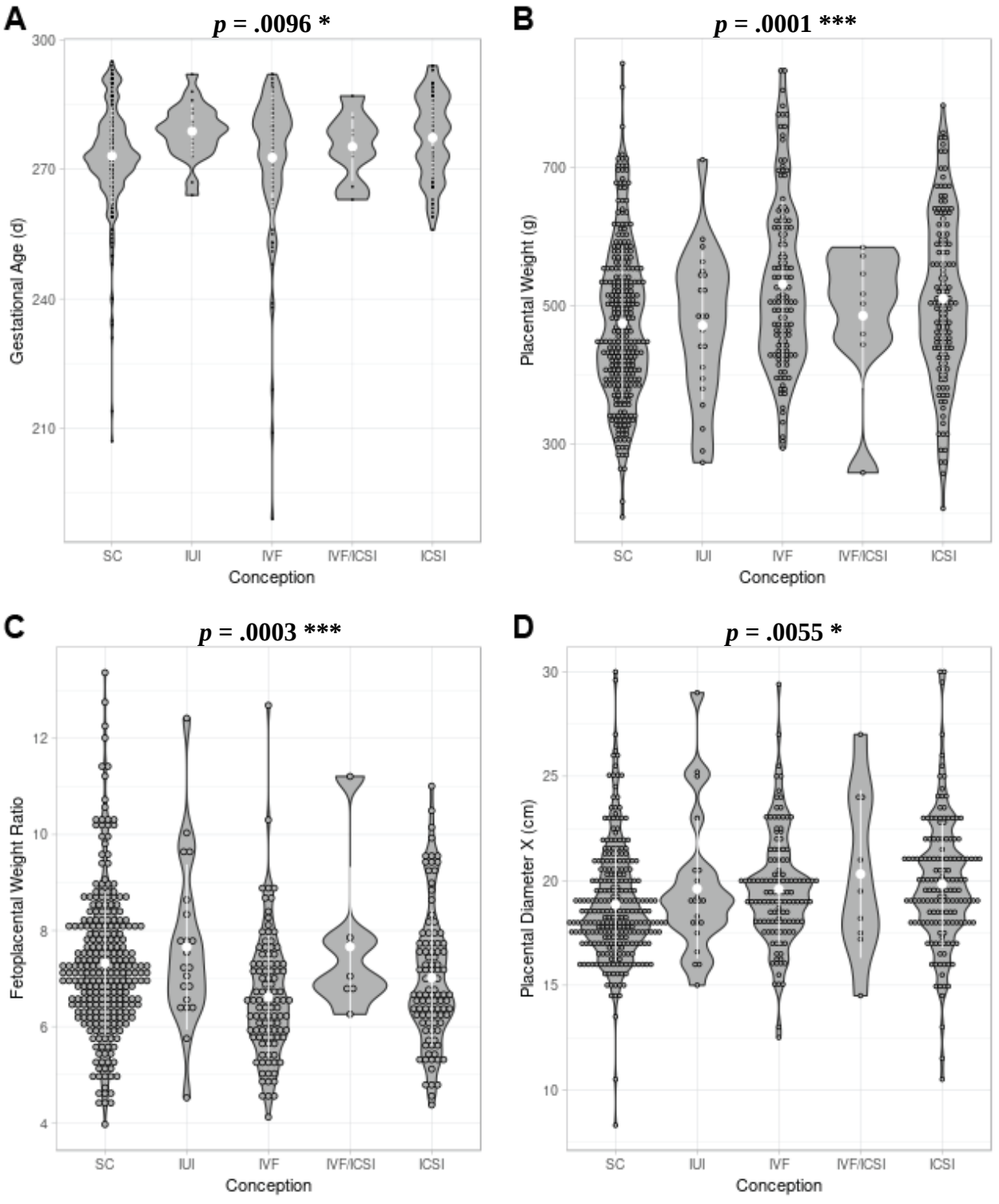
Table 2.2. Clinical information for singleton pregnancies. Table summarizing mean maternal BMI, mean maternal age (y), and percentage of female births, IUGR births, LGA births, and SGA births per conception mode among singleton births. Asterisks denote significance in ANOVA/Kruskal-Wallis H test. * $p \leq .050$, ** $p \leq .010$, *** $p \leq .001$. Superscript letters denote significance in pairwise Tukey HSD/Mann-Whitney U tests. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

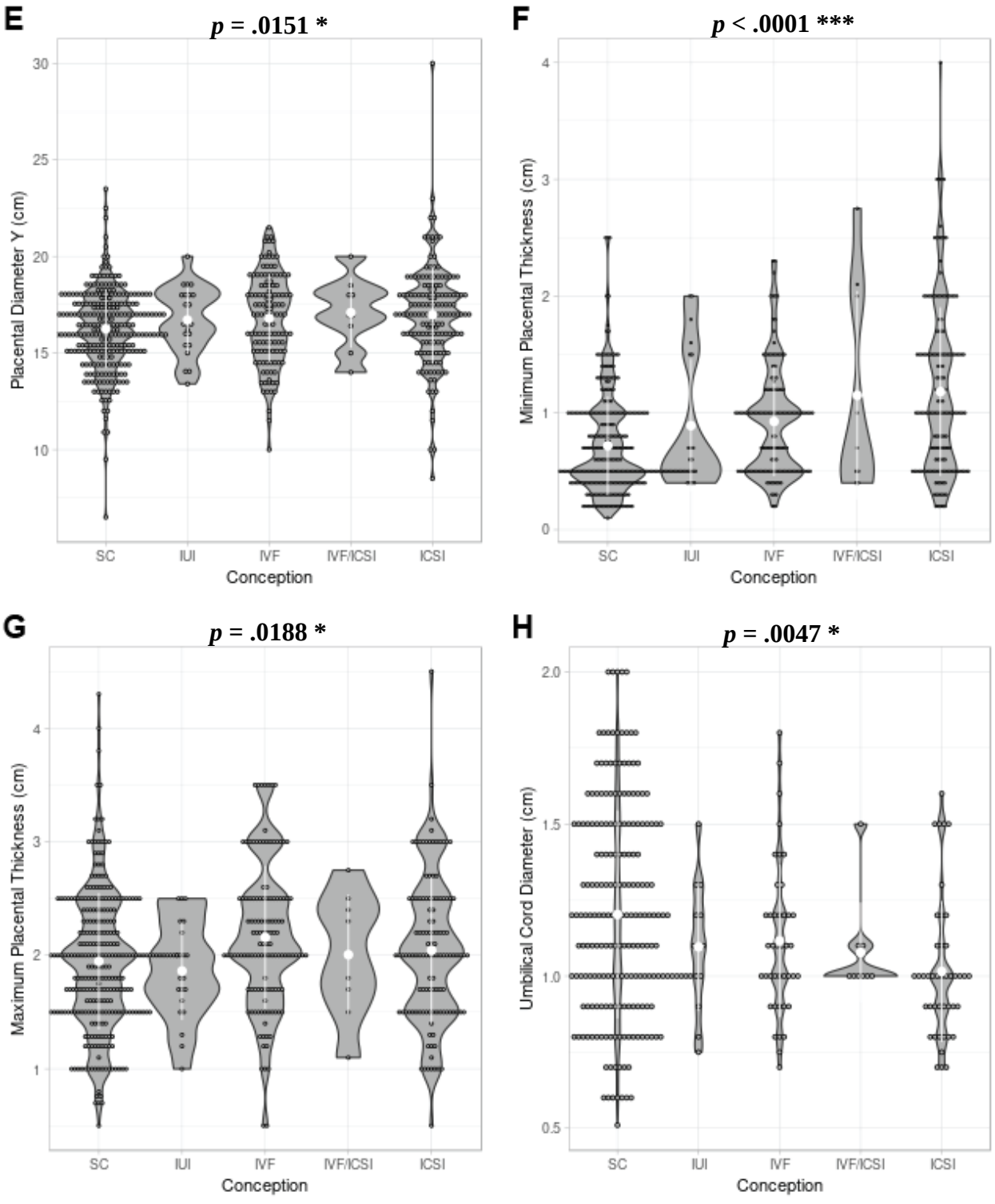
2.3.2 Conception

Based on the specific mode of conception, mean values were calculated for each observed variable and are presented below in Table 2.3. Significant differences were seen in gestational age (p = .0096), placental weight (p = .0001), FPR (p = .0003), placental x diameter (p = .0055), placental y diameter (p = .0151), minimum placental thickness (p < .0001), maximum placental thickness (p = .0188), umbilical cord diameter (p = .0047), and unadjusted body length (p = .0010). Violin plots for these variables can be seen in Figure 2.1.

	SC	IUI	IVF	IVF/ICSI	ICSI	*p* value
n (*N* = 700)	320	25	155	9	191	
Gestational age (days ± SD)	273 ± 11 ^{a}	279 ± 6 ^{ab}	273 ± 16 ^{ab}	275 ± 8 ^{ab}	277 ± 9 ^{b}	.0096 *
Birth weight (percentile ± SD)	49 ± 28	47 ± 33	50 ± 28	60 ± 30	51 ± 25	.7150
Body length (percentile ± SD)	60 ± 26	60 ± 27	62 ± 27	68 ± 35	66 ± 24	.2940
Head circumference (percentile ± SD)	55 ± 28	53 ± 34	50 ± 31	55 ± 33	50 ± 32	.4620
Apgar @ 1 min (± SD)	8.6 ± 1.2	8.7 ± 0.8	8.3 ± 1.2	8.1 ± 1.1	8.3 ± 1.3	.0828
Apgar @ 5 min (± SD)	9.1 ± 0.6	9.1 ± 0.4	9.0 ± 0.4	8.9 ± 0.8	9.1 ± 0.7	.5320
Placental weight (g ± SD)	475 ± 111 ^{a}	471 ± 108 ^{ab}	531 ± 121 ^{b}	485 ± 104 ^{ab}	510 ± 118 ^{b}	.0001 ***
F:P weight ratio (± SD)	7.3 ± 1.5 ^{a}	7.7 ± 1.7 ^{a}	6.6 ± 1.3 ^{b}	7.7 ± 1.7 ^{ab}	7.0 ± 1.4 ^{ab}	.0003 ***
X diameter (cm ± SD)	18.9 ± 2.7 ^{a}	19.6 ± 3.3 ^{ab}	19.6 ± 2.6 ^{ab}	20.3 ± 4.0 ^{ab}	19.8 ± 3.0 ^{b}	.0055 *
Y diameter (cm ± SD)	16.3 ± 2.0 ^{a}	16.7 ± 1.7 ^{ab}	16.8 ± 2.3 ^{ab}	17.1 ± 1.8 ^{ab}	17.0 ± 2.5 ^{b}	.0151 *
Y:X diameter ratio (± SD)	0.87 ± 0.09	0.87 ± 0.11	0.86 ± 0.10	0.86 ± 0.10	0.86 ± 0.10	.9580
Min thickness (cm ± SD)	0.7 ± 0.4 ^{a}	0.9 ± 0.6 ^{abc}	0.9 ± 0.5 ^{b}	1.1 ± 0.9 ^{abc}	1.2 ± 0.7 ^{c}	< .0001 ***
Max thickness (cm ± SD)	1.9 ± 0.6 ^{a}	1.9 ± 0.4 ^{ab}	2.2 ± 0.6 ^{b}	2.0 ± 0.5 ^{ab}	2.0 ± 0.6 ^{ab}	.0188 *
Cord diameter (cm ± SD)	1.2 ± 0.3 ^{a}	1.1 ± 0.2 ^{ab}	1.1 ± 0.3 ^{ab}	1.1 ± 0.2 ^{ab}	1.0 ± 0.2 ^{b}	.0047 *
Birth weight (g ± SD)	3279 ± 593	3440 ± 592	3344 ± 683	3532 ± 332	3444 ± 466	.0902
Body length (cm ± SD)	50.5 ± 2.7 ^{a}	51.4 ± 2.2 ^{ab}	50.8 ± 3.1 ^{a}	51.9 ± 3.0 ^{ab}	51.8 ± 2.3 ^{b}	.0010 **
Head circumference (cm ± SD)	34.6 ± 1.6	34.7 ± 1.8	34.3 ± 1.9	34.7 ± 1.5	34.7 ± 1.7	.5710

Table 2.3. Singleton birth outcomes and placental metrics by conception. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by conception mode among singleton births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.


A
p = .0096 *
Gestational Age (d)
300
270
240
210
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
B
p = .0001 ***
Placental Weight (g)
700
500
300
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
C
p = .0003 ***
Fetoplacental Weight Ratio
12
10
8
6
4
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
D
p = .0055 *
Placental Diameter X (cm)
30
25
20
15
10
SC
IUI
IVF
IVF/ICSI
ICSI
Conception


E
p = .0151 *
Placental Diameter Y (cm)
30
25
20
15
10
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
F
p < .0001 ***
Minimum Placental Thickness (cm)
4
3
2
1
0
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
G
p = .0188 *
Maximum Placental Thickness (cm)
4
3
2
1
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
H
p = .0047 *
Umbilical Cord Diameter (cm)
2.0
1.5
1.0
0.5
SC
IUI
IVF
IVF/ICSI
ICSI
Conception

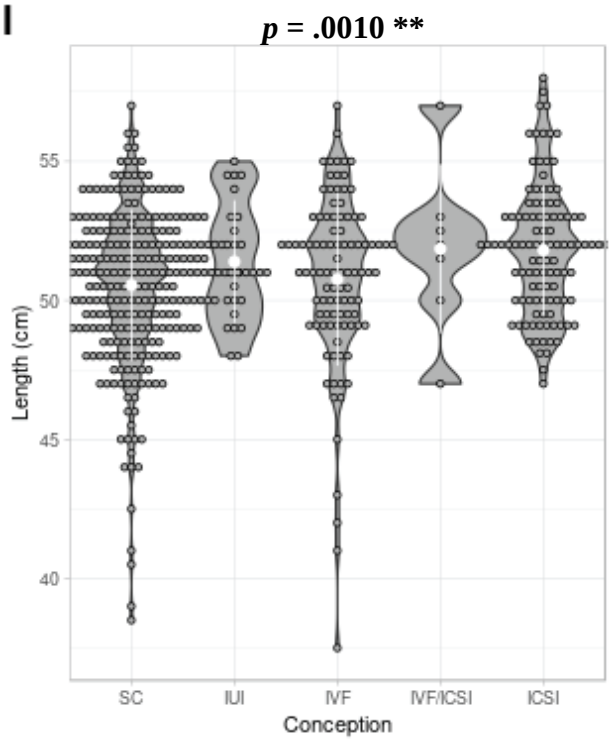


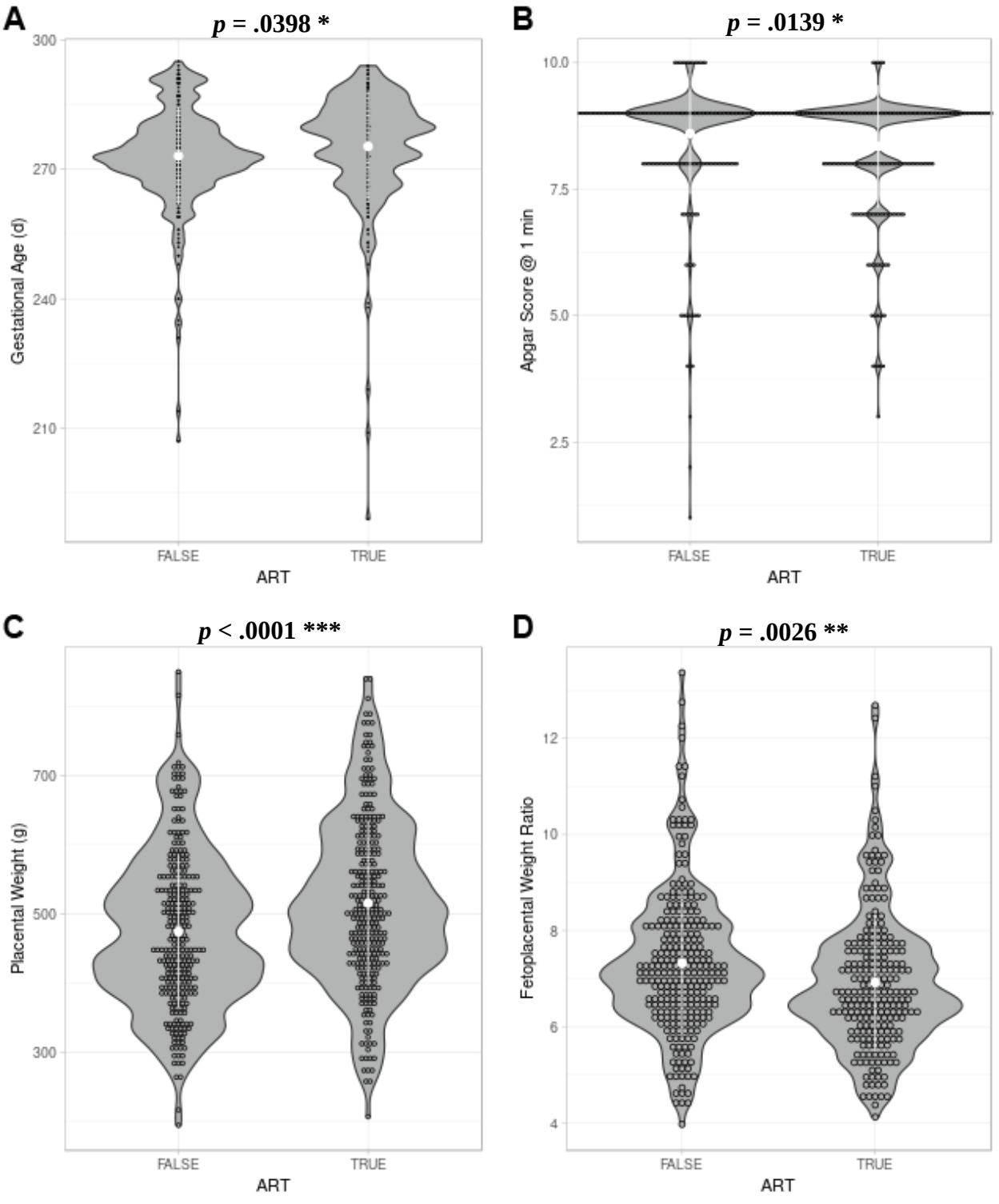
Figure 2.1. Singleton birth outcomes and placental metrics by conception. Violin plots depicting distribution of birth outcomes and placental metrics by mode of conception for each variable that was significantly different in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Placental weight (g). **C**) Fetoplacental weight ratio. **D**) Placental x diameter (cm). **E**) Placental y diameter (cm). **F**) Minimum placental thickness (cm). **G**) Maximum placental thickness (cm). **H**) Umbilical cord diameter (cm). **I**) Unadjusted body length (cm).

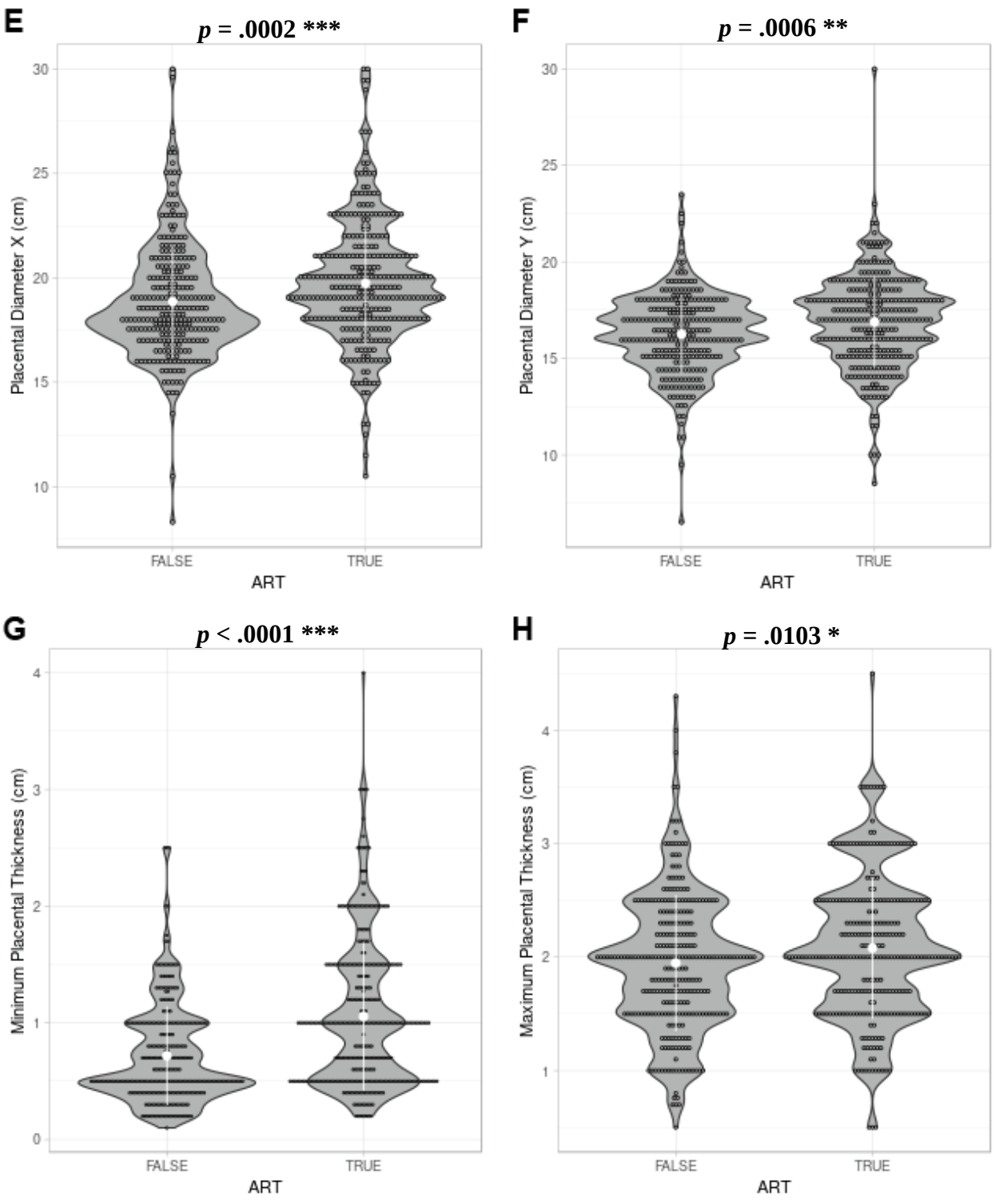
2.3.3 ART

Based on whether or not any ART was used, mean values were calculated for each observed variable and are presented below in Table 2.4. Significant differences were seen in gestational age ($p = .0398$), Apgar score at one minute ($p = .0139$), placental weight ($p < .0001$), FPR ($p = .0026$), placental x diameter ($p = .0002$), placental y diameter ($p = .0006$), minimum placental thickness ($p < .0001$), maximum placental thickness ($p = .0103$), umbilical cord diameter ($p = .0004$), unadjusted birth weight ($p = .0152$), and unadjusted body length ($p = .0011$). Violin plots for these variables can be seen in Figure 2.2.

	SC	ART	*p* value
n (*N* = 700)	320	380	
Gestational age (days ± SD)	273 ± 11	275 ± 13	.0398 *
Birth weight (percentile ± SD)	49 ± 28	51 ± 27	.4007
Body length (percentile ± SD)	60 ± 26	64 ± 26	.0780
Head circumference (percentile ± SD)	55 ± 28	51 ± 31	.0731
Apgar @ 1 min (± SD)	8.6 ± 1.2	8.3 ± 1.2	.0139 *
Apgar @ 5 min (± SD)	9.1 ± 0.6	9.0 ± 0.6	.1598
Placental weight (g ± SD)	475 ± 111	516 ± 119	< .0001 ***
F:P weight ratio (± SD)	7.3 ± 1.5	6.9 ± 1.4	.0026 **
X diameter (cm ± SD)	18.9 ± 2.7	19.7 ± 2.9	.0002 ***
Y diameter (cm ± SD)	16.3 ± 2.0	16.9 ± 2.3	.0006 **
Y:X diameter ratio (± SD)	0.87 ± 0.09	0.86 ± 0.10	.4431
Min thickness (cm ± SD)	0.7 ± 0.4	1.1 ± 0.6	< .0001 ***
Max thickness (cm ± SD)	1.9 ± 0.6	2.1 ± 0.6	.0103 *
Cord diameter (cm ± SD)	1.2 ± 0.3	1.1 ± 0.2	.0004 **
Birth weight (g ± SD)	3279 ± 593	3402 ± 581	.0152 *
Body length (cm ± SD)	50.5 ± 2.7	51.3 ± 2.7	.0011 **
Head circumference (cm ± SD)	34.6 ± 1.6	34.6 ± 1.8	.8199

Table 2.4. Singleton birth outcomes and placental metrics by ART. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of ART among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.


A
p = .0398 *
Gestational Age (d)
300
270
240
210
FALSE
TRUE
ART
B
p = .0139 *
Apgar Score @ 1 min
10.0
7.5
5.0
2.5
FALSE
TRUE
ART
C
p < .0001 ***
Placental Weight (g)
700
500
300
FALSE
TRUE
ART
D
p = .0026 **
Fetoplacental Weight Ratio
12
10
8
6
4
FALSE
TRUE
ART


E
p = .0002 ***
Placental Diameter X (cm)
30
25
20
15
10
FALSE
TRUE
ART
F
p = .0006 **
Placental Diameter Y (cm)
30
25
20
15
10
FALSE
TRUE
ART
G
p < .0001 ***
Minimum Placental Thickness (cm)
4
3
2
1
0
FALSE
TRUE
ART
H
p = .0103 *
Maximum Placental Thickness (cm)
4
3
2
1
FALSE
TRUE
ART

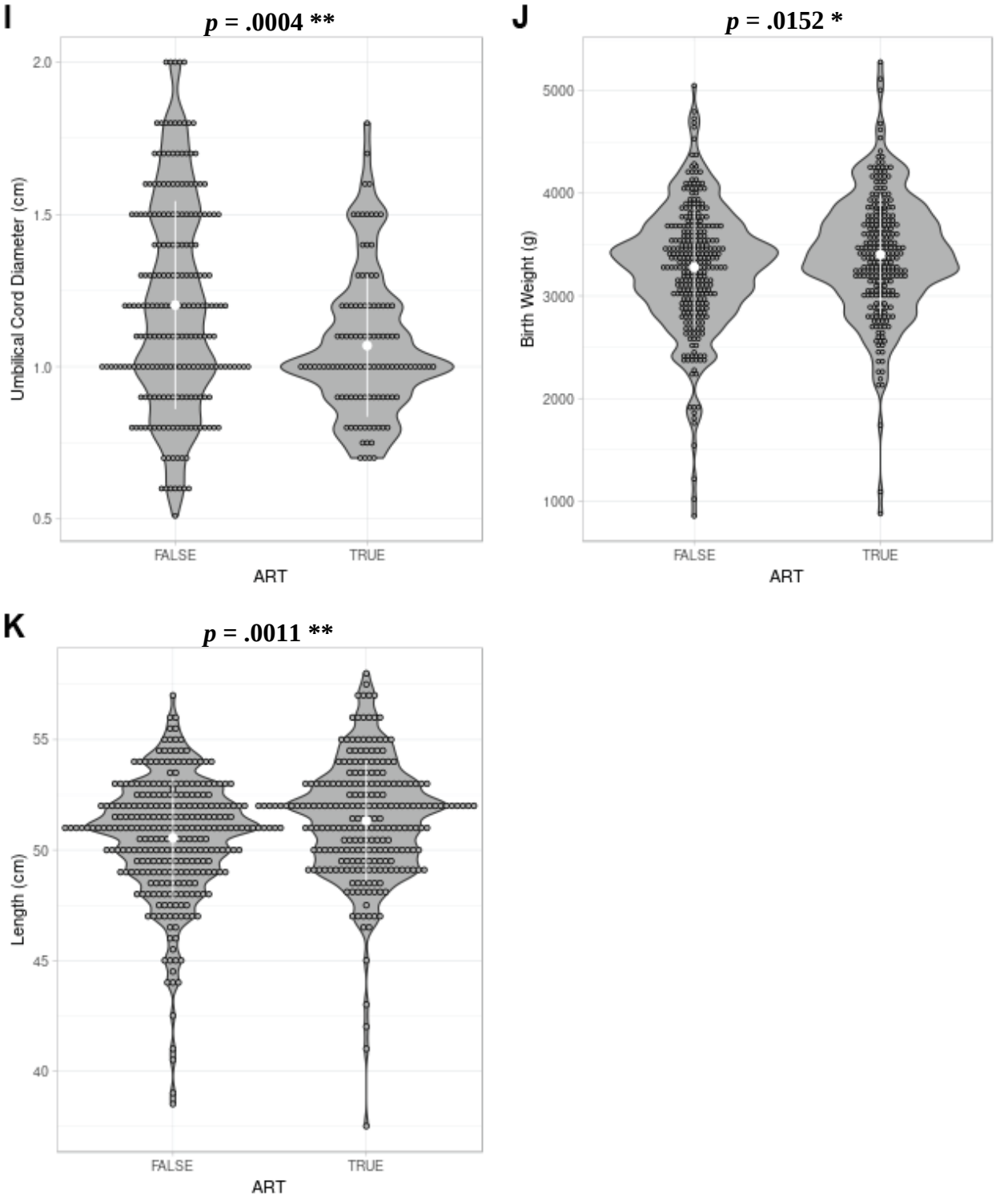


Figure 2.2. Singleton birth outcomes and placental metrics by ART. Violin plots depicting distribution of birth outcomes and placental metrics by use of ART for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean

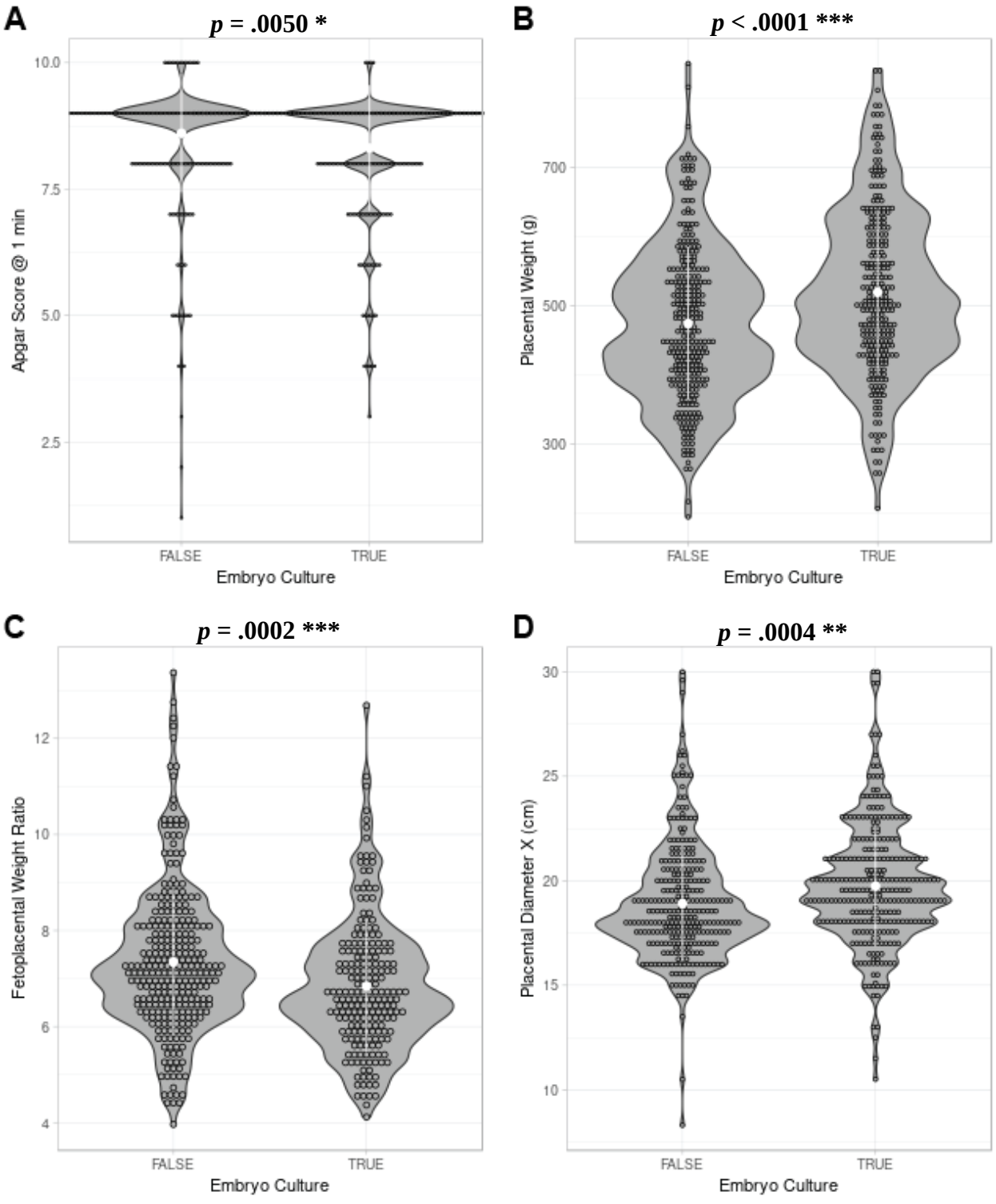
values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Apgar score at 1 minute. **C**) Placental weight (g). **D**) Fetoplacental weight ratio. **E**) Placental x diameter (cm). **F**) Placental y diameter (cm). **G**) Minimum placental thickness (cm). **H**) Maximum placental thickness (cm). **I**) Umbilical cord diameter (cm). **J**) Unadjusted birth weight (g). **K**) Unadjusted body length (cm).

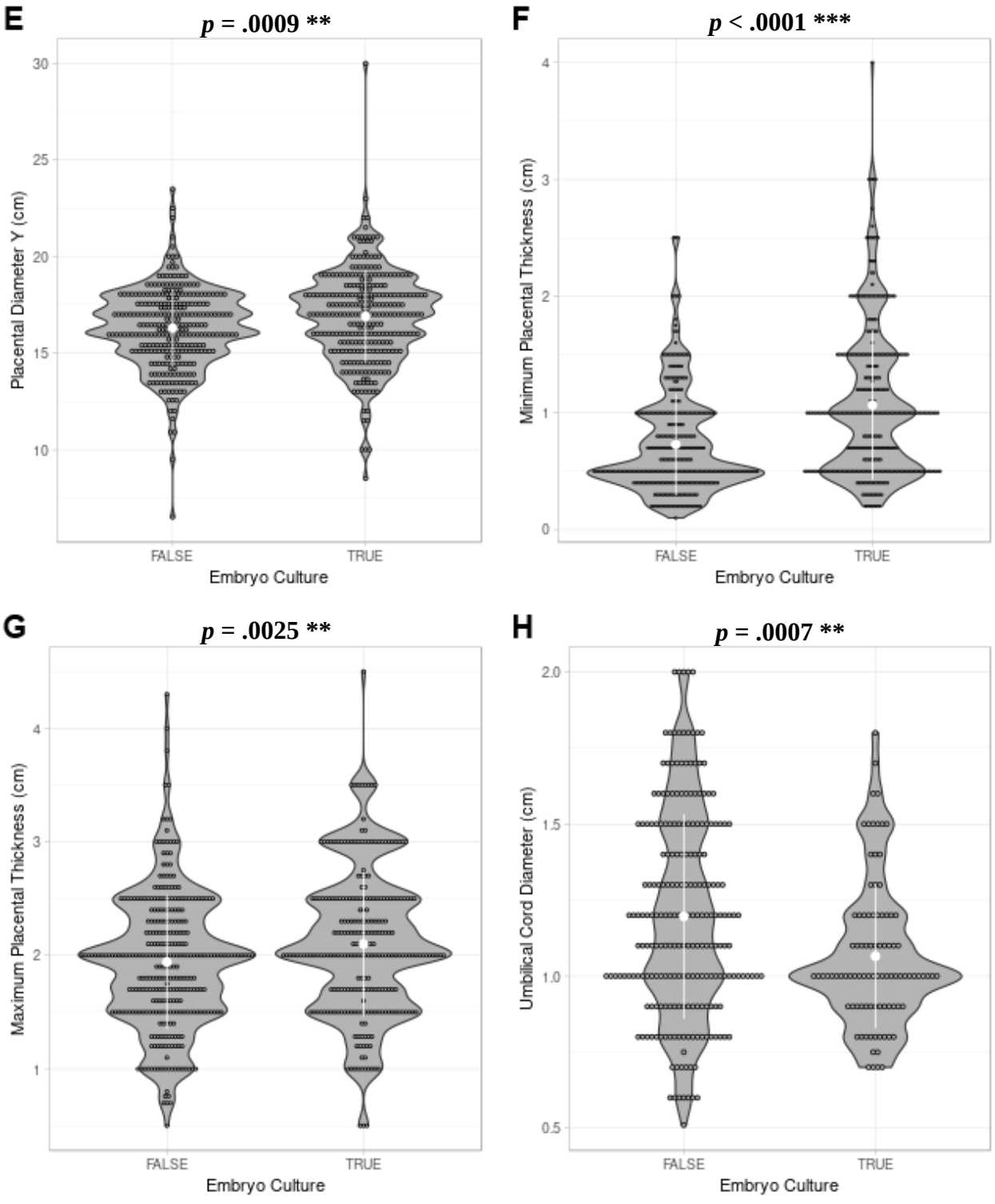
2.3.4 *In vitro* embryo culture

Based on whether or not the embryo was cultured *in vitro*, mean values were calculated for each observed variable and are presented below in Table 2.5. Significant differences were seen in Apgar score at one minute ($p = .0050$), placental weight ($p < .0001$), FPR ($p = .0002$), placental x diameter ($p = .0004$), placental y diameter ($p = .0009$), minimum placental thickness ($p < .0001$), maximum placental thickness ($p = .0025$), umbilical cord diameter ($p = .0007$), unadjusted birth weight ($p = .0357$), and unadjusted body length ($p = .0035$). Violin plots for these variables can be seen in Figures 2.3.

	No *in vitro* culture	*In vitro* culture	*p* value
n (*N* = 700)	345	355	
Gestational age (days ± SD)	274 ± 11	275 ± 14	.2144
Birth weight (percentile ± SD)	48 ± 28	51 ± 26	.2823
Body length (percentile ± SD)	60 ± 26	64 ± 26	.0505
Head circumference (percentile ± SD)	55 ± 29	50 ± 31	.0716
Apgar @ 1 min (± SD)	8.6 ± 1.2	8.3 ± 1.2	.0050 *
Apgar @ 5 min (± SD)	9.1 ± 0.6	9.0 ± 0.6	.1444
Placental weight (g ± SD)	474 ± 111	519 ± 119	< .0001 ***
F:P weight ratio (± SD)	7.3 ± 1.5	6.8 ± 1.4	.0002 ***
X diameter (cm ± SD)	18.9 ± 2.8	19.7 ± 2.9	.0004 **
Y diameter (cm ± SD)	16.3 ± 2.0	16.9 ± 2.4	.0009 **
Y:X diameter ratio (± SD)	0.87 ± 0.1	0.86 ± 0.1	.4516
Min thickness (cm ± SD)	0.7 ± 0.4	1.1 ± 0.6	< .0001 ***
Max thickness (cm ± SD)	1.9 ± 0.6	2.1 ± 0.6	.0025 **
Cord diameter (cm ± SD)	1.2 ± 0.3	1.1 ± 0.2	.0007 **
Birth weight (g ± SD)	3291 ± 594	3398 ± 581	.0357 *
Body length (cm ± SD)	50.6 ± 2.7	51.3 ± 2.8	.0035 *
Head circumference (cm ± SD)	34.6 ± 1.6	34.5 ± 1.8	.6916

Table 2.5. Singleton birth outcomes and placental metrics by *in vitro* culture. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of *in vitro* embryo culturing among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.


A
p = .0050 *
Apgar Score @ 1 min
10.0
7.5
5.0
2.5
FALSE
TRUE
Embryo Culture
B
p < .0001 ***
Placental Weight (g)
700
500
300
FALSE
TRUE
Embryo Culture
C
p = .0002 ***
Fetoplacental Weight Ratio
12
10
8
6
4
FALSE
TRUE
Embryo Culture
D
p = .0004 **
Placental Diameter X (cm)
30
25
20
15
10
FALSE
TRUE
Embryo Culture


E
p = .0009 **
Placental Diameter Y (cm)
30
25
20
15
10
FALSE
TRUE
Embryo Culture
F
p < .0001 ***
Minimum Placental Thickness (cm)
4
3
2
1
0
FALSE
TRUE
Embryo Culture
G
p = .0025 **
Maximum Placental Thickness (cm)
4
3
2
1
FALSE
TRUE
Embryo Culture
H
p = .0007 **
Umbilical Cord Diameter (cm)
2.0
1.5
1.0
0.5
FALSE
TRUE
Embryo Culture

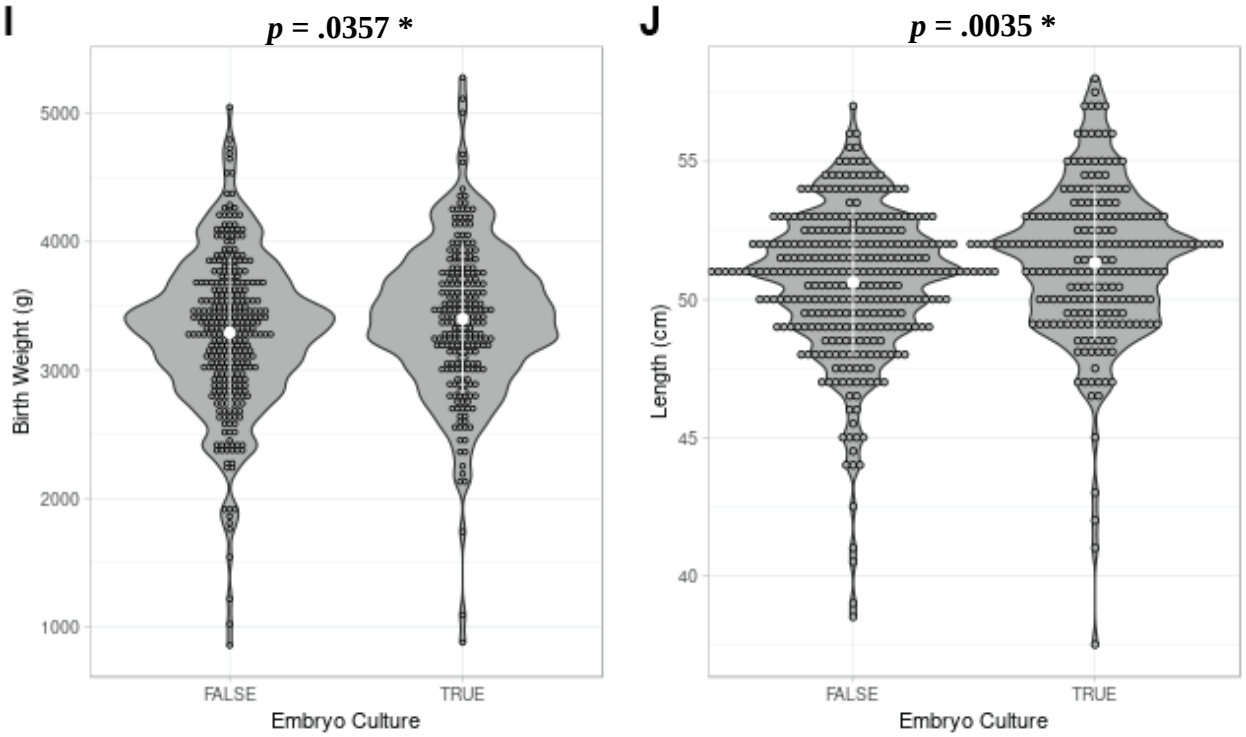


Figure 2.3. Singleton birth outcomes and placental metrics by embryo culture. Violin plots depicting distribution of birth outcomes and placental metrics by use of *in vitro* embryo culture for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Apgar score at 1 minute. **B**) Placental weight (g). **C**) Fetoplacental weight ratio. **D**) Placental x diameter (cm). **E**) Placental y diameter (cm). **F**) Minimum placental thickness (cm). **G**) Maximum placental thickness (cm). **H**) Umbilical cord diameter (cm). **I**) Unadjusted birth weight (g). **J**) Unadjusted body length (cm).

2.3.5 Infertility

Based on which parent was diagnosed with infertility, mean values were calculated for each observed variable and are presented below in Table 2.6. The "Neither" group is comprised of both pregnancies in which no ART was used and pregnancies in which ART was used for reasons other than infertility. The "NA" group is comprised of pregnancies in which an ART was used, but fertility status was unknown in this database and was therefor not included in the statistical comparisons. Significant differences were seen in placental x diameter (p = .0009), placental y diameter (p = .0066), placental diameter ratio (p = .0283), and minimum placental thickness (p = .0058). Violin plots for these variables can be seen in Figure 2.4.

	Neither	Male	Female	Both	NA	*p* value
n (*N* = 700)	330	14	16	5	335	
Gestational age (days ± SD)	273 ± 11	271 ± 25	272 ± 13	277 ± 6	276 ± 12	.8240
Birth weight (percentile ± SD)	49 ± 28	63 ± 31	45 ± 28	62 ± 23	49 ± 26	.2300
Body length (percentile ± SD)	60 ± 26	65 ± 28	60 ± 32	78 ± 17	64 ± 25	.4300
Head circumference (percentile ± SD)	56 ± 28	59 ± 36	48 ± 30	56 ± 30	49 ± 31	.7910
Apgar @ 1 min (± SD)	8.6 ± 1.2	8.1 ± 1.8	8.5 ± 0.8	7.8 ± 0.8	8.4 ± 1.2	.2550
Apgar @ 5 min (± SD)	9.1 ± 0.6	8.7 ± 0.9	9.1 ± 0.3	9.0 ± 0.0	9.1 ± 0.6	.1080
Placental weight (g ± SD)	476 ± 111	499 ± 118	447 ± 103	541 ± 128	518 ± 120	.3950
F:P weight ratio (± SD)	7.3 ± 1.5	7.9 ± 1.6	7.1 ± 0.9	6.9 ± 1.6	6.9 ± 1.4	.4940
X diameter (cm ± SD)	18.9 ± 2.8 [a]	19.9 ± 2.3 [a]	19.8 ± 2.2 [a]	23.8 ± 4.7 [b]	19.6 ± 2.9	.0009 **
Y diameter (cm ± SD)	16.3 ± 2.1	17.8 ± 1.7	17.7 ± 2.1	17.7 ± 1.8	16.8 ± 2.4	.0066 *
Y:X diameter ratio (± SD)	0.87 ± 0.10 [a]	0.90 ± 0.07 [a]	0.89 ± 0.05 [a]	0.76 ± 0.11 [b]	0.86 ± 0.10	.0283 *
Min thickness (cm ± SD)	0.7 ± 0.4 [a]	1.1 ± 0.5 [b]	1.0 ± 0.3 [ab]	0.7 ± 0.2 [ab]	1.1 ± 0.6	.0058 *
Max thickness (cm ± SD)	2.0 ± 0.6	2.0 ± 0.3	2.4 ± 0.7	2.2 ± 0.5	2.1 ± 0.6	.1320
Cord diameter (cm ± SD)	1.2 ± 0.3	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	.0621
Birth weight (g ± SD)	3297 ± 601	3507 ± 957	3161 ± 655	3578 ± 264	3387 ± 543	.3660
Body length (cm ± SD)	50.6 ± 2.7	52.2 ± 3.1	50.3 ± 3.7	52.6 ± 1.3	51.3 ± 2.6	.0927
Head circumference (cm ± SD)	34.6 ± 1.6	35.2 ± 1.9	34.1 ± 1.6	34.9 ± 1.5	34.5 ± 1.8	.4030

Table 2.6. Singleton birth outcomes and placental metrics by infertility. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by source of infertility among singleton births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.

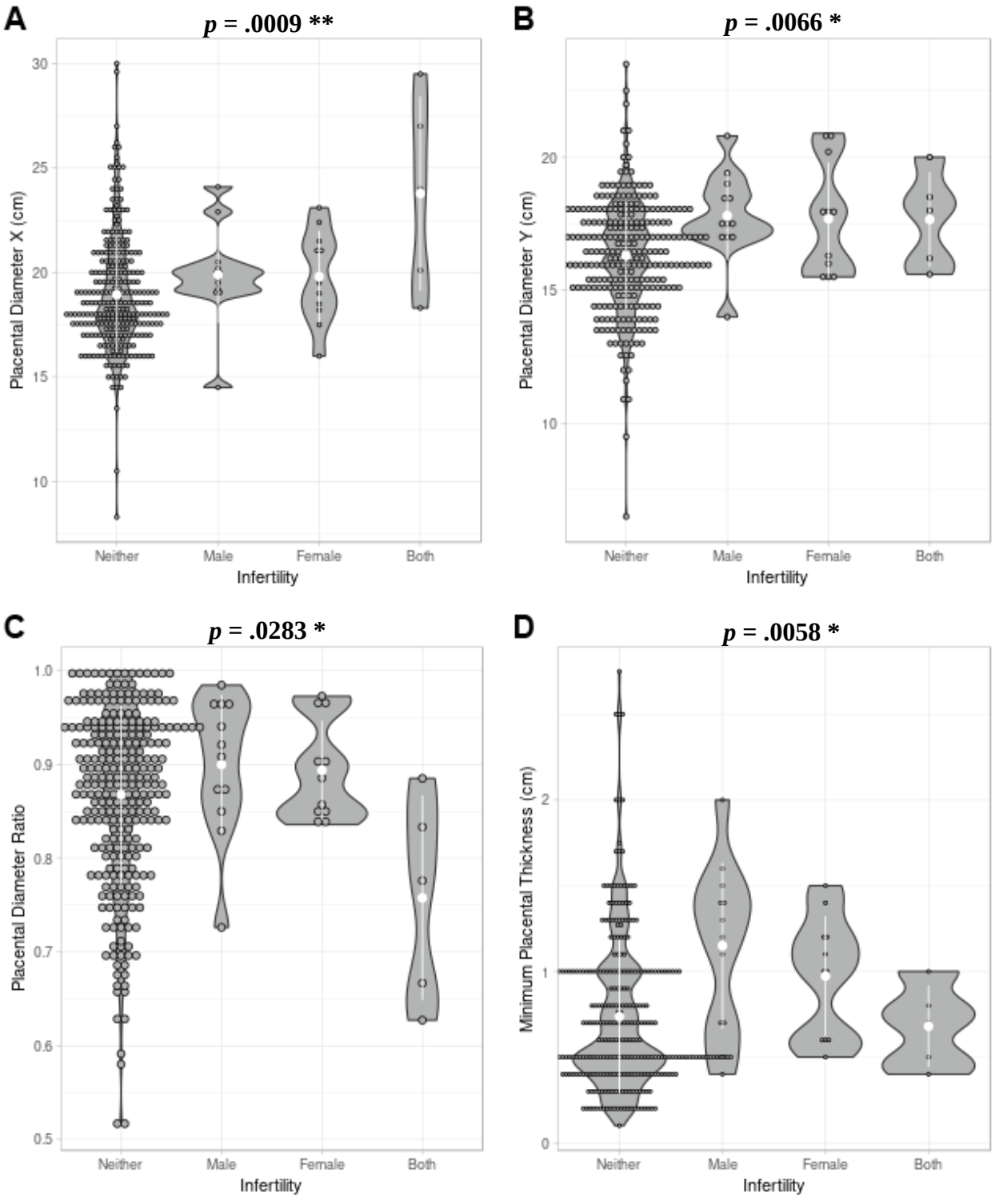


Figure 2.4. Singleton birth outcomes and placental metrics by infertility. Violin plots depicting distribution of birth outcomes and placental metrics by source of infertility for each variable that was significantly different in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote

mean values, and white lines denote standard deviation ranges. **A**) Placental x diameter (cm). **B**) Placental y diameter (cm). **C**) Placental y:x diameter ratio. **D**) Minimum placental thickness (cm).

2.3.6 Gravidity

Based on the mother's gravidity, mean values were calculated for each observed variable and are presented below in Table 2.7. The "NA" group is comprised of pregnancies for which the mother's gravidity was unknown in this database and was therefor not included in the statistical comparisons. Significant differences were seen in head circumference percentile (p = .0044), Apgar score at one minute (p = .0239), and umbilical cord diameter (p = .0238). Violin plots for these variables can be seen in Figure 2.5.

	1	2	3	4	NA	*p* value
n (*N* = 700)	64	79	46	13	498	
Gestational age (days ± SD)	274 ± 16	270 ± 12	271 ± 10	272 ± 11	276 ± 11	.2110
Birth weight (percentile ± SD)	48 ± 28	55 ± 27	51 ± 32	47 ± 35	48 ± 27	.5760
Body length (percentile ± SD)	57 ± 30	61 ± 25	64 ± 25	59 ± 32	62 ± 26	.5860
Head circumference (percentile ± SD)	45 ± 30 ^{a}	59 ± 29 ^{b}	62 ± 26 ^{b}	68 ± 28 ^{ab}	52 ± 30	.0044 *
Apgar @ 1 min (± SD)	8.3 ± 1.5 ^{a}	8.6 ± 0.9 ^{ab}	8.9 ± 0.4 ^{b}	8.9 ± 0.5 ^{ab}	8.4 ± 1.3	.0239 *
Apgar @ 5 min (± SD)	9.0 ± 0.4	9.1 ± 0.6	9.2 ± 0.4	9.2 ± 0.4	9.1 ± 0.6	.2680
Placental weight (g ± SD)	488 ± 123	449 ± 107	475 ± 121	502 ± 138	506 ± 115	.2370
F:P weight ratio (± SD)	7.4 ± 1.7	7.9 ± 1.7	7.4 ± 1.6	7.0 ± 1.1	6.9 ± 1.3	.1990
X diameter (cm ± SD)	19.3 ± 3.2	19.2 ± 2.7	19.5 ± 3.1	19.0 ± 2.0	19.3 ± 2.8	.9690
Y diameter (cm ± SD)	16.6 ± 2.3	16.5 ± 2.3	16.3 ± 2.1	16.6 ± 1.8	16.6 ± 2.2	.9580
Y:X diameter ratio (± SD)	0.87 ± 0.08	0.86 ± 0.11	0.85 ± 0.10	0.88 ± 0.11	0.87 ± 0.10	.7490
Min thickness (cm ± SD)	0.8 ± 0.5	0.8 ± 0.5	0.9 ± 0.6	1.0 ± 0.5	0.9 ± 0.6	.4610
Max thickness (cm ± SD)	2.1 ± 0.5	2.1 ± 0.6	2.3 ± 0.6	2.4 ± 0.5	1.9 ± 0.6	.3840
Cord diameter (cm ± SD)	1.1 ± 0.3 ^{a}	1.2 ± 0.3 ^{b}	1.2 ± 0.3 ^{ab}	1.2 ± 0.3 ^{ab}	1.2 ± 0.3	.0238 *
Birth weight (g ± SD)	3288 ± 694	3288 ± 561	3280 ± 649	3353 ± 775	3361 ± 565	.9880
Body length (cm ± SD)	50.3 ± 3.9	50.3 ± 2.4	50.8 ± 2.7	50.5 ± 2.9	51.2 ± 2.5	.8000
Head circumference (cm ± SD)	34.0 ± 2.2	34.6 ± 1.5	34.7 ± 1.4	35.2 ± 1.8	34.7 ± 1.7	.0800

Table 2.7. Singleton birth outcomes and placental metrics by maternal gravidity. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by maternal gravidity among singleton births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.

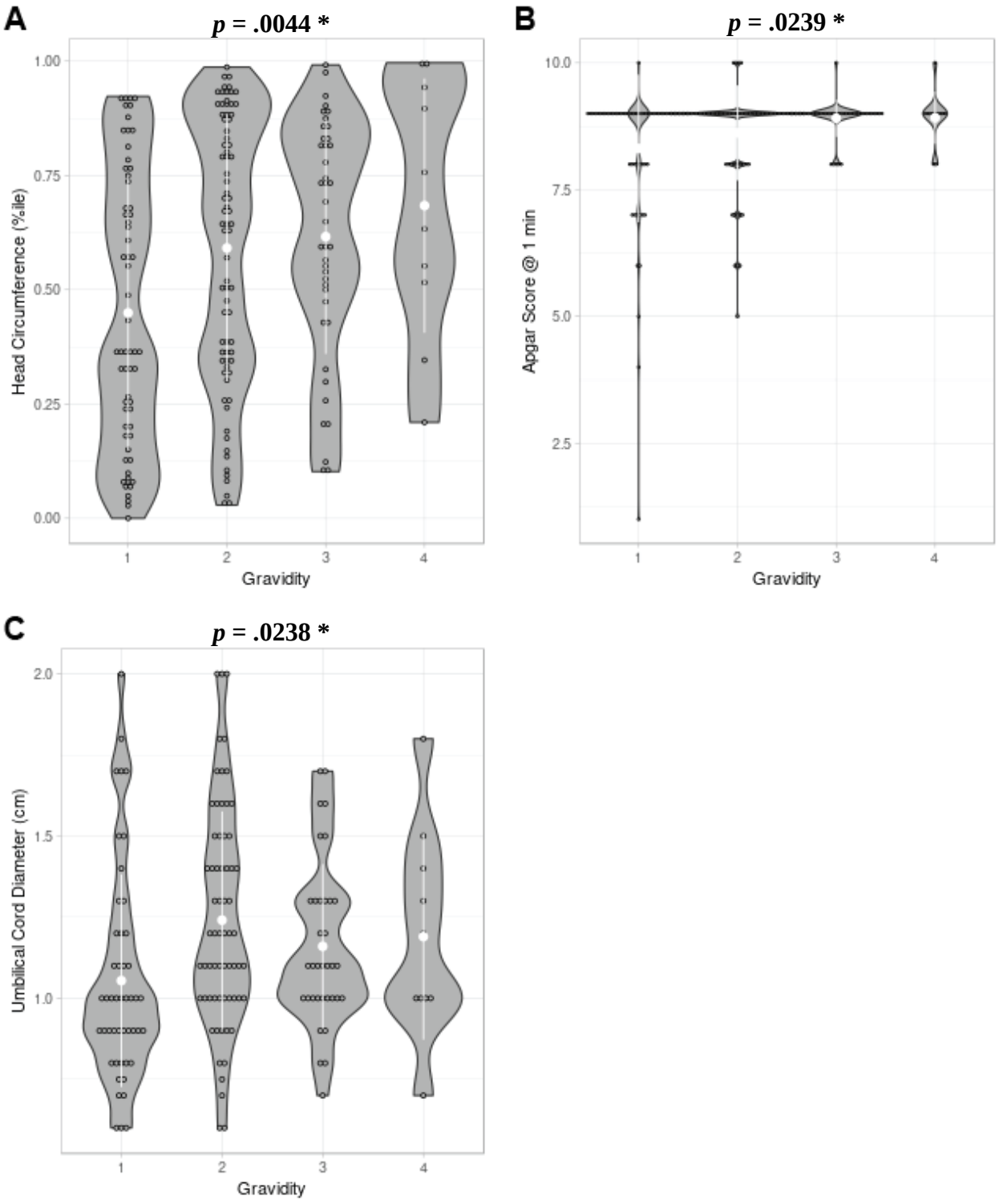


Figure 2.5. Singleton birth outcomes and placental metrics by gravidity. Violin plots depicting distribution of birth outcomes and placental metrics by maternal gravidity for each variable that was significantly different in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote

mean values, and white lines denote standard deviation ranges. **A**) Head circumference (%ile). **B**) Apgar score at 1 minute. **C**) Umbilical cord diameter (cm).

2.3.7 Embryo transfer

Based on whether a fresh or frozen embryo was transferred, mean values were calculated for each observed variable and are presented below in Table 2.8. The "NA" group is comprised of both pregnancies in which no embryo transfer occurred (56%) and pregnancies in which an embryo transfer occurred, but fresh or frozen status was unknown in this database (44%) and was therefor not included in the statistical comparisons. Significant differences were seen in head circumference percentile (p = .0260) and placental weight (p = .0380). Violin plots for these variables can be seen in Figure 2.6.

	Fresh	Frozen	NA	*p* value
n (*N* = 700)	47	38	615	
Gestational age (days ± SD)	272 ± 14	273 ± 21	274 ± 11	.8343
Birth weight (percentile ± SD)	49 ± 27	61 ± 28	49 ± 27	.0622
Body length (percentile ± SD)	61 ± 29	67 ± 26	61 ± 26	.3703
Head circumference (percentile ± SD)	44 ± 32	61 ± 31	54 ± 29	.0260 *
Apgar @ 1 min (± SD)	8.4 ± 1.3	8.4 ± 0.9	8.5 ± 1.2	.7853
Apgar @ 5 min (± SD)	8.9 ± 0.8	9.0 ± 0.5	9.1 ± 0.6	.5634
Placental weight (g ± SD)	465 ± 98	520 ± 113	497 ± 118	.0380 *
F:P weight ratio (± SD)	7.3 ± 1.6	7.1 ± 1.0	7.1 ± 1.5	.5687
X diameter (cm ± SD)	19.6 ± 2.7	20.7 ± 3.1	19.2 ± 2.8	.1204
Y diameter (cm ± SD)	17.2 ± 1.9	17.8 ± 1.8	16.5 ± 2.2	.1965
Y:X diameter ratio (± SD)	0.88 ± 0.07	0.87 ± 0.10	0.86 ± 0.10	.4905
Min thickness (cm ± SD)	0.9 ± 0.4	1.1 ± 0.6	0.9 ± 0.6	.0510
Max thickness (cm ± SD)	2.1 ± 0.5	2.3 ± 0.5	2.0 ± 0.6	.0859
Cord diameter (cm ± SD)	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.3	.6872
Birth weight (g ± SD)	3232 ± 564	3495 ± 866	3334 ± 567	.1097
Body length (cm ± SD)	50.5 ± 3.2	51.5 ± 3.7	50.9 ± 2.6	.2171
Head circumference (cm ± SD)	34.0 ± 2.0	34.9 ± 2.4	34.6 ± 1.6	.0741

Table 2.8. Singleton birth outcomes and placental metrics by embryo transfer. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by type of embryo transfer among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

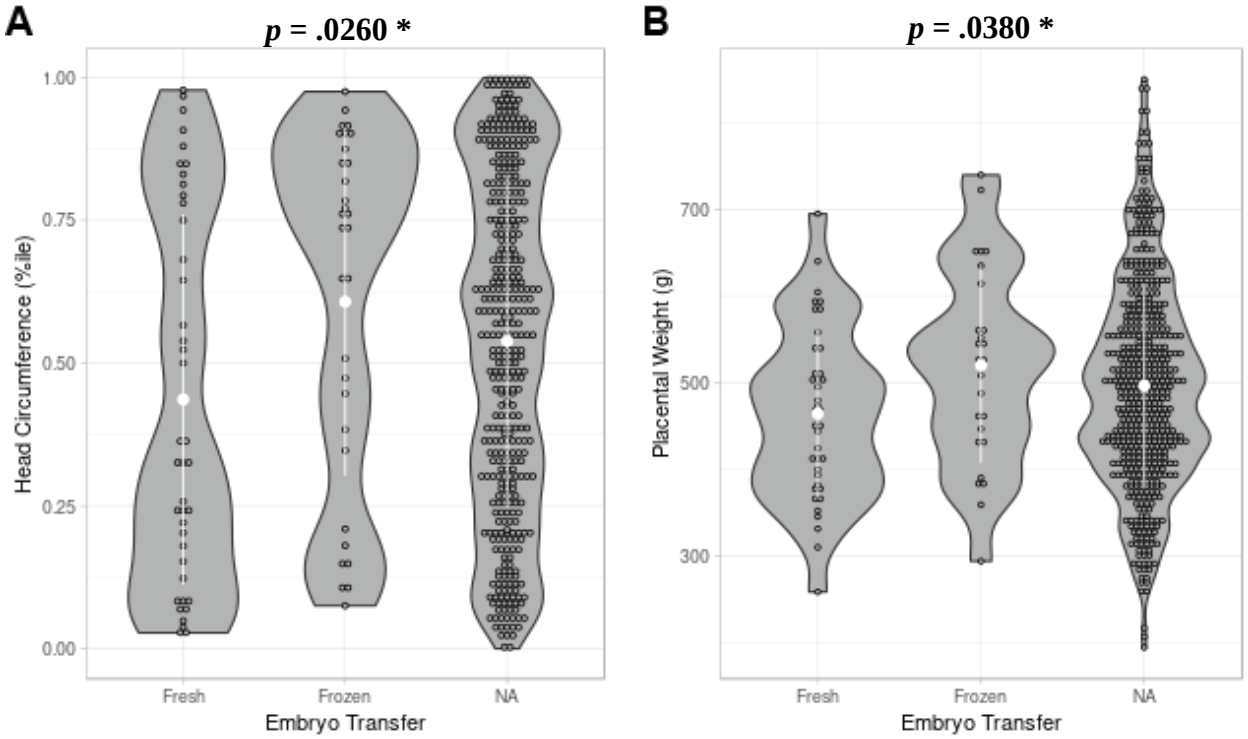


Figure 2.6. Singleton birth outcomes and placental metrics by embryo transfer. Violin plots depicting distribution of birth outcomes and placental metrics by type of embryo transfer for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Head circumference (%ile). **B**) Placental weight (g).

2.3.8 Sperm donor

Based on whether or not a sperm donor was used, mean values were calculated for each observed variable and are presented below in Table 2.9. A significant difference was seen in head circumference percentile (p = .0126). A violin plot for this variable can be seen in Figure 2.7.

	No sperm donor	Sperm donor	*p* value
n (*N* = 700)	693	7	
Gestational age (days ± SD)	274 ± 12	270 ± 12	.3577
Birth weight (percentile ± SD)	49 ± 27	69 ± 31	.0553
Body length (percentile ± SD)	61 ± 26	71 ± 28	.3567
Head circumference (percentile ± SD)	53 ± 30	81 ± 18	.0126 *
Apgar @ 1 min (± SD)	8.5 ± 1.2	8.0 ± 1.5	.2886
Apgar @ 5 min (± SD)	9.1 ± 0.6	8.9 ± 0.4	.3152
Placental weight (g ± SD)	495 ± 117	513 ± 118	.6851
F:P weight ratio (± SD)	7.1 ± 1.5	7.1 ± 0.5	.9325
X diameter (cm ± SD)	19.3 ± 2.9	21.2 ± 2.0	.1156
Y diameter (cm ± SD)	16.6 ± 2.2	17.5 ± 2.3	.3157
Y:X diameter ratio (± SD)	0.87 ± 0.1	0.83 ± 0.1	.3537
Min thickness (cm ± SD)	0.9 ± 0.6	1.2 ± 0.6	.1772
Max thickness (cm ± SD)	2.0 ± 0.6	2.1 ± 0.8	.6612
Cord diameter (cm ± SD)	1.2 ± 0.3	1.1 ± 0.2	.5326
Birth weight (g ± SD)	3332 ± 589	3610 ± 704	.2162
Body length (cm ± SD)	50.9 ± 2.7	51.1 ± 2.8	.8116
Head circumference (cm ± SD)	34.6 ± 1.7	35.6 ± 1.2	.1227

Table 2.9. Singleton birth outcomes and placental metrics by sperm donor. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of sperm donor among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

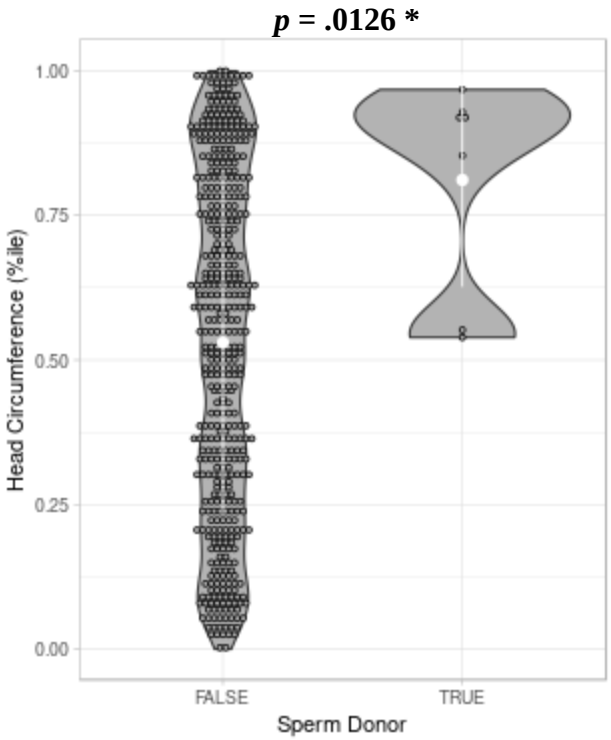


Figure 2.7. Singleton birth outcomes and placental metrics by sperm donor. Violin plot depicting distribution of body length (%ile), the only variable that was significantly different in Tukey HSD test, by use of a sperm donor. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges.

2.3.9 Egg donor

Based on whether or not an egg donor was used, mean values were calculated for each observed variable and are presented below in Table 2.10. Significant differences were seen in gestational age (p = .0054), Apgar score at five minutes (p = .0479), maximum placental thickness (p = .0200), and unadjusted body length (p = .0340). Violin plots for these variables can be seen in Figure 2.8.

	No egg donor	Egg donor	*p* value
n (*N* = 700)	695	5	
Gestational age (days ± SD)	274 ± 12	255 ± 14	.0054 *
Birth weight (percentile ± SD)	50 ± 28	36 ± 26	.3968
Body length (percentile ± SD)	62 ± 26	37 ± 35	.1074
Head circumference (percentile ± SD)	54 ± 30	40 ± 34	.4392
Apgar @ 1 min (± SD)	8.5 ± 1.2	7.5 ± 2.4	.1028
Apgar @ 5 min (± SD)	9.1 ± 0.6	8.5 ± 1.7	.0479 *
Placental weight (g ± SD)	496 ± 117	410 ± 111	.1402
F:P weight ratio (± SD)	7.1 ± 1.5	7.3 ± 1.5	.8030
X diameter (cm ± SD)	19.3 ± 2.9	19.8 ± 3.8	.7640
Y diameter (cm ± SD)	16.6 ± 2.2	17.0 ± 1.8	.7304
Y:X diameter ratio (± SD)	0.87 ± 0.10	0.87 ± 0.08	.9284
Min thickness (cm ± SD)	0.9 ± 0.6	1.2 ± 0.1	.3726
Max thickness (cm ± SD)	2.0 ± 0.6	2.7 ± 0.5	.0200 *
Cord diameter (cm ± SD)	1.2 ± 0.3	1.0 ± 0.2	.1859
Birth weight (g ± SD)	3340 ± 589	2784 ± 559	.0603
Body length (cm ± SD)	50.9 ± 2.7	48.0 ± 4.6	.0340 *
Head circumference (cm ± SD)	34.6 ± 1.7	33.2 ± 2.5	.1033

Table 2.10. Singleton birth outcomes and placental metrics by egg donor. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of egg donor among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

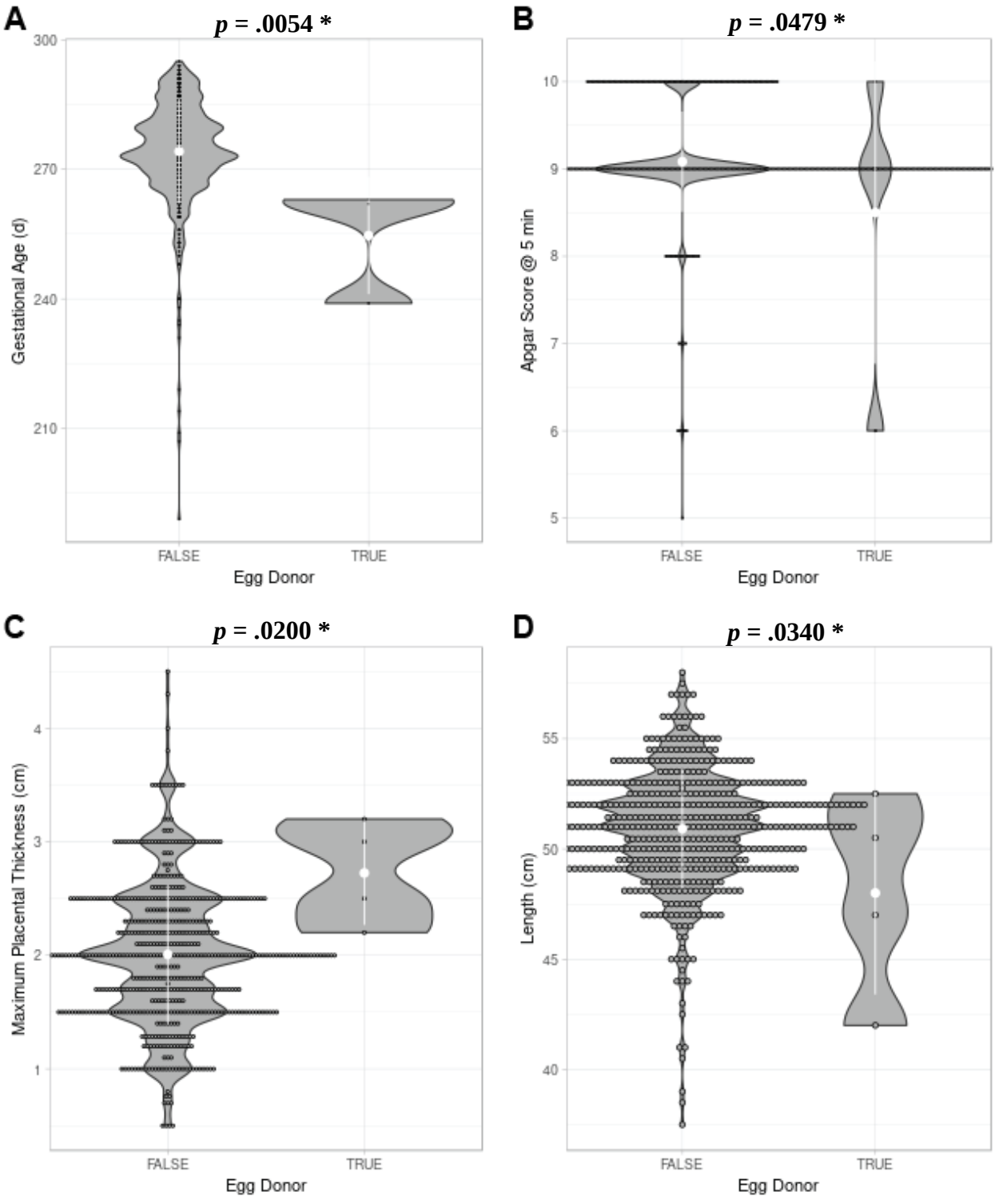


Figure 2.8. Singleton birth outcomes and placental metrics by egg donor. Violin plots depicting distribution of birth outcomes and placental metrics by use of egg donor for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote

mean values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Apgar score at 5 minutes. **C**) Maximum placental thickness (cm). **D**) Unadjusted body length (cm).

2.3.10 Surrogate

Based on whether or not a surrogate mother was used, mean values were calculated for each observed variable and are presented below in Table 2.11. Significant differences were seen in birth weight percentile (p = .0211), unadjusted birth weight (p = .0017), unadjusted body length (p = .0341), and unadjusted head circumference (p = .0445). Violin plots for these variables can be seen in Figure 2.9.

	No surrogate	Surrogate	*p* value
n (*N* = 700)	697	3	
Gestational age (days ± SD)	274 ± 12	284 ± 9	.2709
Birth weight (percentile ± SD)	49 ± 27	94 ± 7	.0211 *
Body length (percentile ± SD)	61 ± 26	94 ± 6	.0777
Head circumference (percentile ± SD)	53 ± 30	87 ± 14	.1054
Apgar @ 1 min (± SD)	8.5 ± 1.2	9.0 ± 0.0	.5435
Apgar @ 5 min (± SD)	9.1 ± 0.6	9.0 ± 0.0	.8491
Placental weight (g ± SD)	495 ± 117	560 ± NA	.5838
F:P weight ratio (± SD)	7.1 ± 1.5	NA ± NA	NA
X diameter (cm ± SD)	19.3 ± 2.9	24.4 ± NA	.0756
Y diameter (cm ± SD)	16.6 ± 2.2	19.0 ± NA	.2792
Y:X diameter ratio (± SD)	0.87 ± 0.10	0.78 ± NA	.3728
Min thickness (cm ± SD)	0.9 ± 0.6	1.3 ± NA	.4792
Max thickness (cm ± SD)	2.0 ± 0.6	3.2 ± NA	.0533
Cord diameter (cm ± SD)	1.2 ± 0.3	0.9 ± NA	.4144
Birth weight (g ± SD)	3331 ± 585	4638 ± 909	.0017 **
Body length (cm ± SD)	50.9 ± 2.7	55.0 ± 2.8	.0341 *
Head circumference (cm ± SD)	34.6 ± 1.7	37.0 ± 2.1	.0445 *

Table 2.11. Singleton birth outcomes and placental metrics by surrogate. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of gestational surrogate among singleton births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

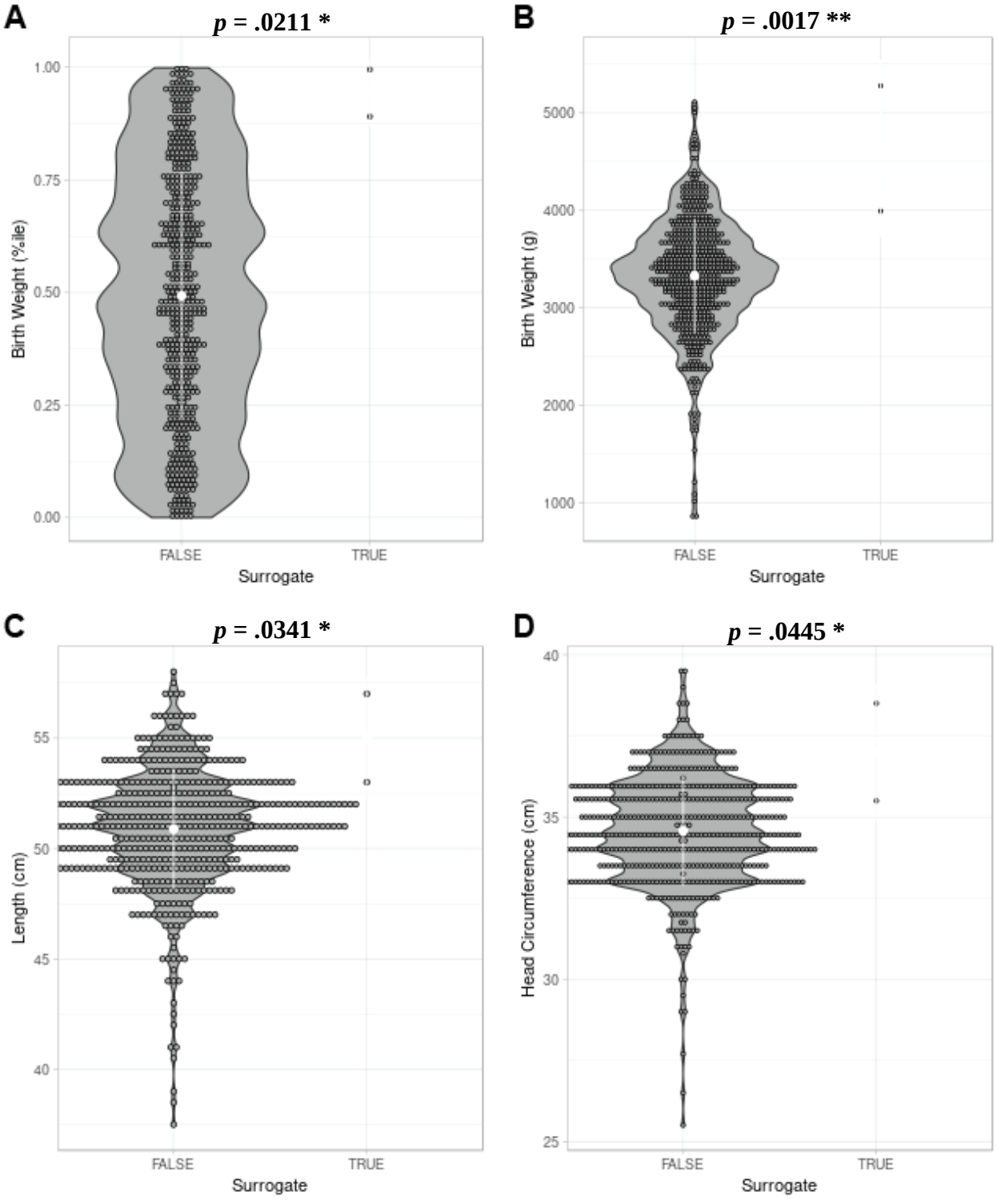


Figure 2.9. Singleton birth outcomes and placental metrics by surrogate. Violin plots depicting distribution of birth outcomes and placental metrics by use of gestational surrogate for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots

denote mean values, and white lines denote standard deviation ranges. **A**) Birth weight (%ile). **B**) Unadjusted birth weight (g). **C**) Unadjusted body length (cm). **D**) Unadjusted head circumference (cm).

2.4 Results (twins)

2.4.1 Clinical information

Similar to what was seen in the singleton births, pregnancies in the IVF (p = .024) and ICSI (p = .009) groups had significantly higher maternal age than the SC group. There were significantly fewer IUGR births in the IVF (p < .001), IUI (p = .014), IVF/ICSI (p = .042), and ICSI (p < .001) groups than the SC group. The IVF and IVF/ICSI groups also had significantly more IUGR births than the ICSI group (p = .003) and (p < .001) (Table 2.12). Percentages reported for female, IUGR, LGA, and SGA births were calculated using the cases in each group for which the data was known, not necessarily all the cases in each group.

	SC	IUI	IVF	IVF/ICSI	ICSI	p value
n (N = 170)	19	4	72	8	67	
Mean Mat. Age (years ± SD)	29 ± 6 ^{a}	38 ± 3 ^{ab}	35 ± 5 ^{b}	33 ± 3 ^{ab}	36 ± 2 ^{b}	.009 **
Mean Mat. BMI (± SD)	27 ± 6	29 ± 10	25 ± 4	27 ± 2	22 ± 3	.221
Female (%)	41 (7/17)	50 (2/4)	54 (32/59)	38 (3/8)	40 (12/30)	.675
IUGR (%)	68 ^{a} (13/19)	0 ^{bc} (0/4)	12 ^{b} (9/72)	25 ^{b} (2/8)	0 ^{c} (0/67)	< .001 ***
LGA (%)	0 (0/17)	0 (0/4)	0 (0/55)	0 (0/6)	0 (0/23)	NA
SGA (%)	35 (6/17)	0 (0/4)	15 (8/55)	50 (3/6)	30 (7/23)	.091

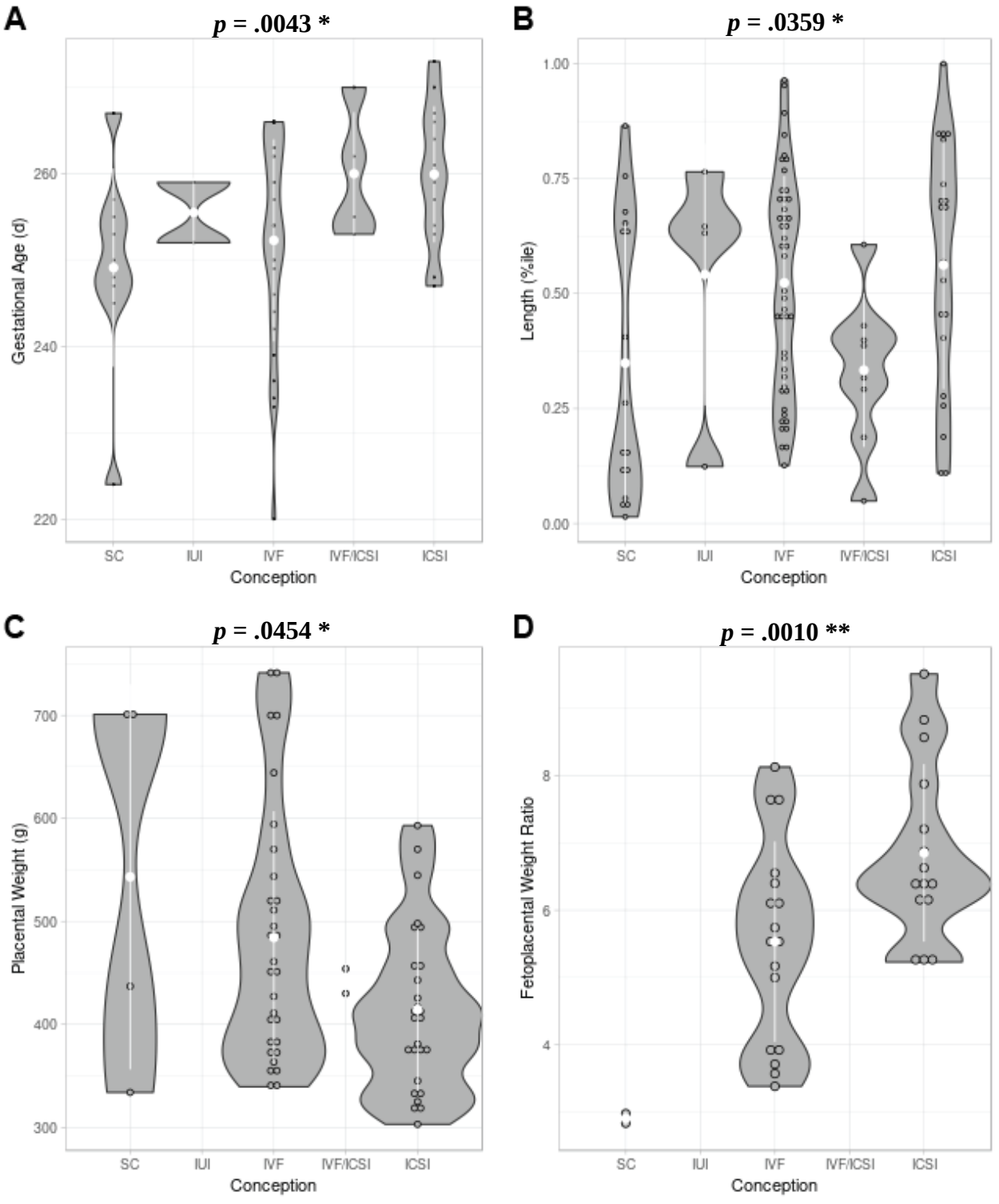
Table 2.12. Clinical information for twin pregnancies. Table summarizing mean maternal BMI, mean maternal age (y), and percentage of female births, IUGR births, LGA births, and SGA births per conception mode among twin births. Asterisks denote significance in ANOVA/Kruskal-Wallis H test. * $p \leq .050$, ** $p \leq .010$, *** $p \leq .001$. Superscript letters denote significance in pairwise Tukey HSD/Mann-Whitney U tests. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

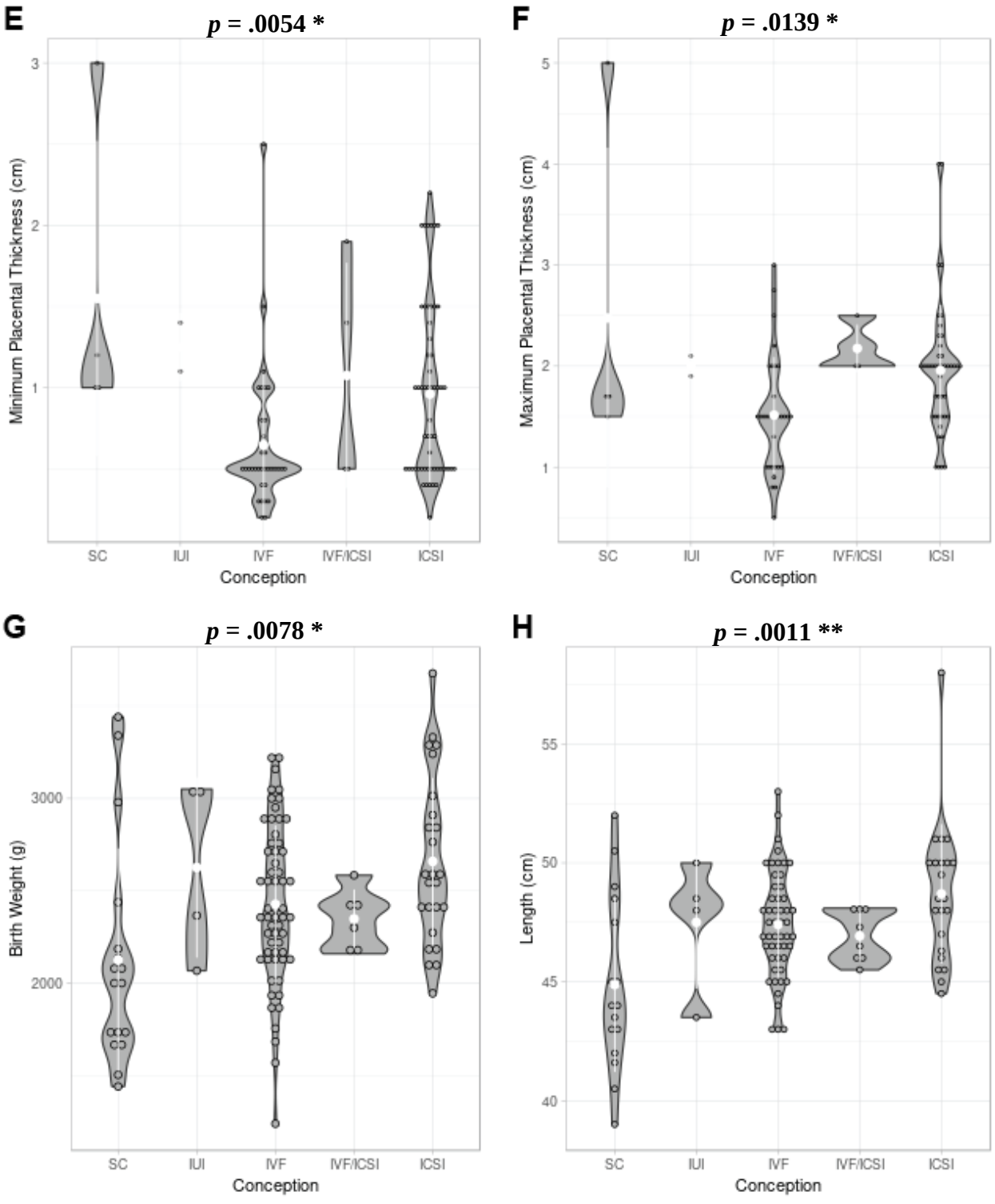
2.4.2 Conception

Based on the specific mode of conception, mean values were calculated for each observed variable and are presented below in Table 2.13. Significant differences were seen in gestational age (p = .0043), body length percentile (p = .0359), placental weight (p = .0454), FPR (p = .0010), minimum placental thickness (p = .0054), maximum placental thickness (p = .0139), unadjusted birth weight (p = .0078), unadjusted body length (p = .0011), and unadjusted head circumference (p = .0002). Violin plots for these variables can be seen in Figure 2.10.

	SC	IUI	IVF	IVF/ICSI	ICSI	*p* value
n (*N* = 170)	19	4	72	8	67	
Gestational age (days ± SD)	249 ± 11 ^{a}	256 ± 4 ^{ab}	252 ± 12 ^{a}	260 ± 7 ^{ab}	260 ± 8 ^{b}	.0043 *
Birth weight (percentile ± SD)	21 ± 23	39 ± 28	32 ± 21	14 ± 13	33 ± 27	.1300
Body length (percentile ± SD)	35 ± 30	54 ± 28	52 ± 23	33 ± 17	56 ± 27	.0359 *
Head circumference (percentile ± SD)	40 ± 27	57 ± 18	61 ± 23	55 ± 31	63 ± 28	.0546
Apgar @ 1 min (± SD)	7.8 ± 1.5	9.0 ± 0.0	8.1 ± 1.7	8.9 ± 0.4	8.7 ± 1.0	.1980
Apgar @ 5 min (± SD)	8.8 ± 0.6	9.0 ± 0.0	8.9 ± 0.7	9.2 ± 0.5	9.0 ± 0.3	.4070
Placental weight (g ± SD)	543 ± 187	NA ± NA	484 ± 123	442 ± 17	414 ± 79	.0454 *
F:P weight ratio (± SD)	2.9 ± 0.1 ^{a}	NA ± NA	5.5 ± 1.5 ^{b}	NA ± NA	6.9 ± 1.3 ^{c}	.0010 **
X diameter (cm ± SD)	18.2 ± 3.4	22.2 ± 4.2	21.4 ± 4.7	19.7 ± 3.0	20.5 ± 3.6	.5320
Y diameter (cm ± SD)	14.2 ± 4.8	16.7 ± 2.2	16.9 ± 3.5	16.9 ± 2.1	16.1 ± 3.4	.5990
Y:X diameter ratio (± SD)	0.76 ± 0.13	0.78 ± 0.24	0.81 ± 0.13	0.88 ± 0.21	0.79 ± 0.13	.7540
Min thickness (cm ± SD)	1.6 ± 1.0 ^{a}	1.2 ± 0.2 ^{ab}	0.6 ± 0.4 ^{b}	1.1 ± 0.7 ^{ab}	1.0 ± 0.5 ^{ab}	.0054 *
Max thickness (cm ± SD)	2.5 ± 1.7 ^{ab}	2.0 ± 0.1 ^{ab}	1.5 ± 0.6 ^{a}	2.2 ± 0.2 ^{ab}	2.0 ± 0.6 ^{b}	.0139 *
Cord diameter (cm ± SD)	NA ± NA	1.0 ± 0.0	0.9 ± 0.1	1.3 ± 0.5	1.0 ± 0.3	.2300
Birth weight (g ± SD)	2128 ± 601 ^{a}	2626 ± 487 ^{ab}	2428 ± 427 ^{ab}	2349 ± 159 ^{ab}	2659 ± 452 ^{b}	.0078 *
Body length (cm ± SD)	44.9 ± 3.7 ^{a}	47.5 ± 2.8 ^{ab}	47.4 ± 2.2 ^{b}	46.9 ± 1.1 ^{ab}	48.7 ± 2.9 ^{b}	.0011 **
Head circumference (cm ± SD)	31.6 ± 1.9 ^{a}	33.1 ± 1.2 ^{ab}	33.0 ± 1.4 ^{b}	33.3 ± 1.3 ^{ab}	34.0 ± 1.3 ^{b}	.0002 ***

Table 2.13. Twin birth outcomes and placental metrics by conception. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by conception mode among twin births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.


A
p = .0043 *
Gestational Age (d)
260
240
220
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
B
p = .0359 *
Length (%ile)
1.00
0.75
0.50
0.25
0.00
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
C
p = .0454 *
Placental Weight (g)
700
600
500
400
300
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
D
p = .0010 **
Fetoplacental Weight Ratio
8
6
4
SC
IUI
IVF
IVF/ICSI
ICSI
Conception


E
p = .0054 *
Minimum Placental Thickness (cm)
3
2
1
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
F
p = .0139 *
Maximum Placental Thickness (cm)
5
4
3
2
1
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
G
p = .0078 *
Birth Weight (g)
3000
2000
SC
IUI
IVF
IVF/ICSI
ICSI
Conception
H
p = .0011 **
Length (cm)
55
50
45
40
SC
IUI
IVF
IVF/ICSI
ICSI
Conception

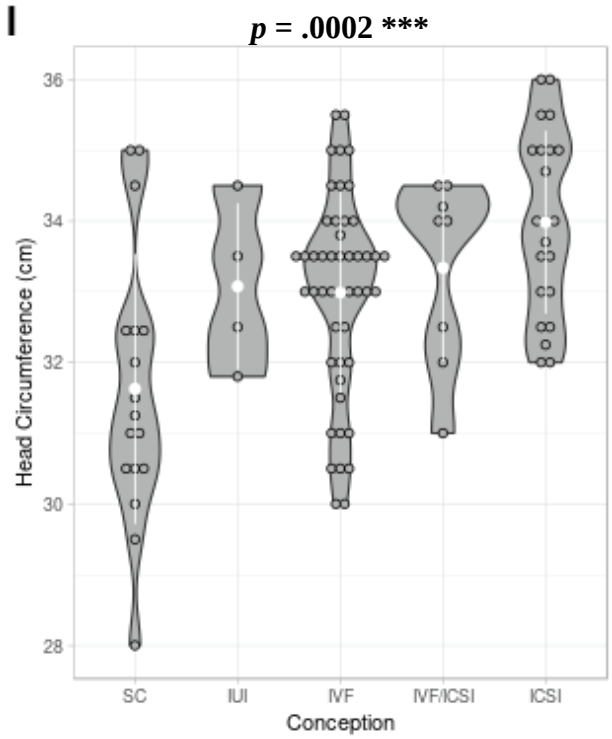


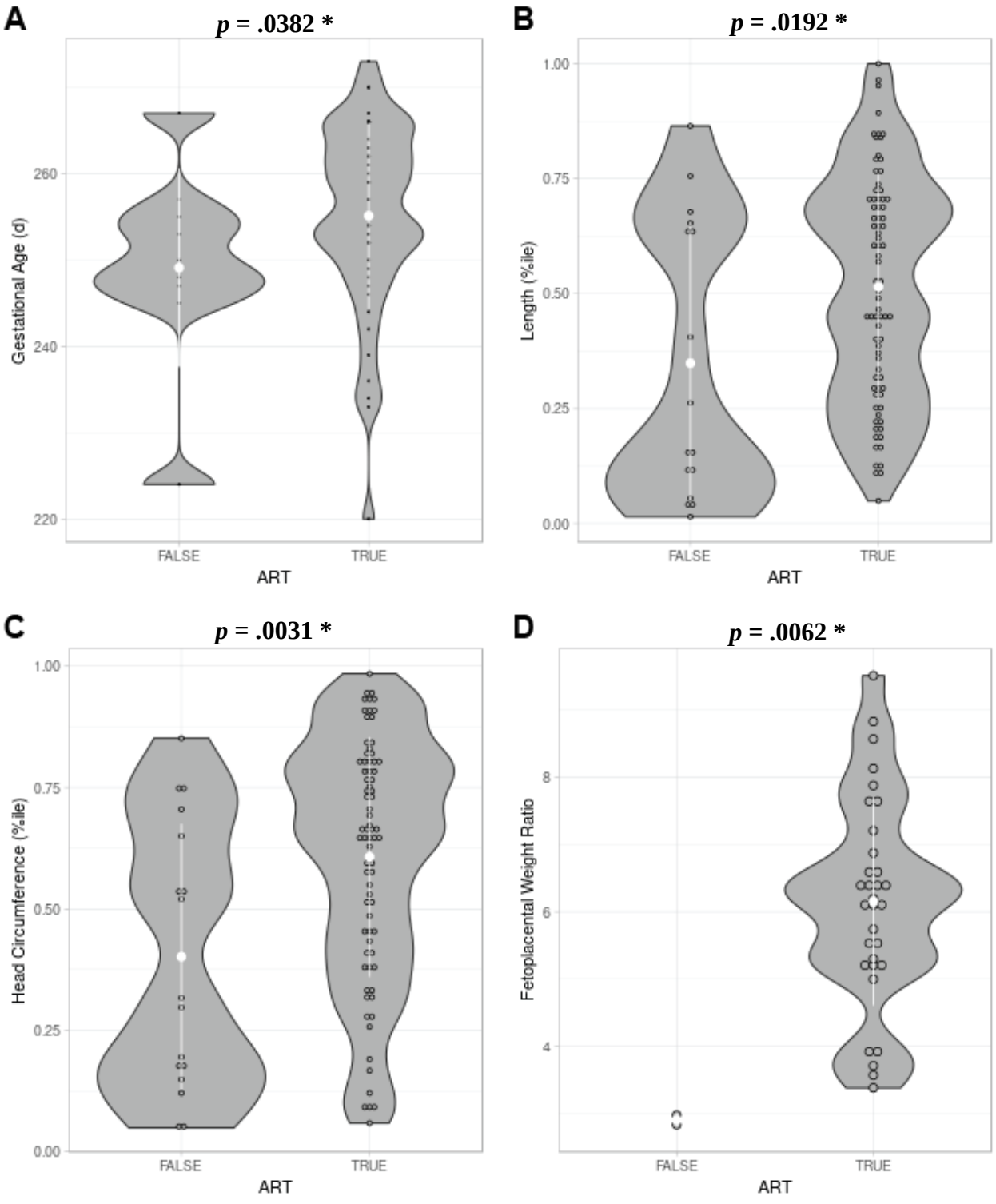
Figure 2.10. Twin birth outcomes and placental metrics by conception. Violin plots depicting distribution of birth outcomes and placental metrics by mode of conception for each variable that was significantly different in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Body length (%ile). **C**) Placental weight (g). **D**) Fetoplacental weight ratio. **E**) Minimum placental thickness (cm). **F**) Maximum placental thickness (cm). **G**) Unadjusted birth weight (g). **H**) Unadjusted body length (cm). **I**) Unadjusted head circumference (cm).

2.4.3 ART

Based on whether or not any ART was used, mean values were calculated for each observed variable and are presented below in Table 2.14. Significant differences were seen in gestational age (p = .0382), body length percentile (p = .0192), head circumference percentile (p = .0031), FPR (p = .0062), minimum placental thickness (p = .0153), unadjusted birth weight (p = .0034), unadjusted body length (p = .0002), and unadjusted head circumference (p = .0001). Violin plots for these variables can be seen in Figure 2.11.

	SC	ART	*p* value
n (*N* = 170)	19	151	
Gestational age (days ± SD)	249 ± 11	255 ± 11	.0382 *
Birth weight (percentile ± SD)	21 ± 23	31 ± 22	.0777
Body length (percentile ± SD)	35 ± 30	51 ± 24	.0192 *
Head circumference (percentile ± SD)	40 ± 27	61 ± 25	.0031 *
Apgar @ 1 min (± SD)	7.8 ± 1.5	8.4 ± 1.5	.1552
Apgar @ 5 min (± SD)	8.8 ± 0.6	8.9 ± 0.6	.3202
Placental weight (g ± SD)	543 ± 187	451 ± 107	.1176
F:P weight ratio (± SD)	2.9 ± 0.1	6.2 ± 1.5	.0062 *
X diameter (cm ± SD)	18.2 ± 3.4	20.8 ± 4.0	.2092
Y diameter (cm ± SD)	14.2 ± 4.8	16.5 ± 3.4	.2105
Y:X diameter ratio (± SD)	0.76 ± 0.13	0.80 ± 0.14	.5877
Min thickness (cm ± SD)	1.6 ± 1.0	0.9 ± 0.5	.0153 *
Max thickness (cm ± SD)	2.5 ± 1.7	1.8 ± 0.6	.0619
Cord diameter (cm ± SD)	NA ± NA	1.1 ± 0.3	NA
Birth weight (g ± SD)	2128 ± 601	2493 ± 433	.0034 *
Body length (cm ± SD)	44.9 ± 3.7	47.7 ± 2.4	.0002 ***
Head circumference (cm ± SD)	31.6 ± 1.9	33.3 ± 1.4	.0001 ***

Table 2.14. Twin birth outcomes and placental metrics by ART. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of ART among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.


A
p = .0382 *
Gestational Age (d)
260
240
220
FALSE
TRUE
ART
B
p = .0192 *
Length (%ile)
1.00
0.75
0.50
0.25
0.00
FALSE
TRUE
ART
C
p = .0031 *
Head Circumference (%ile)
1.00
0.75
0.50
0.25
0.00
FALSE
TRUE
ART
D
p = .0062 *
Fetoplacental Weight Ratio
8
6
4
FALSE
TRUE
ART

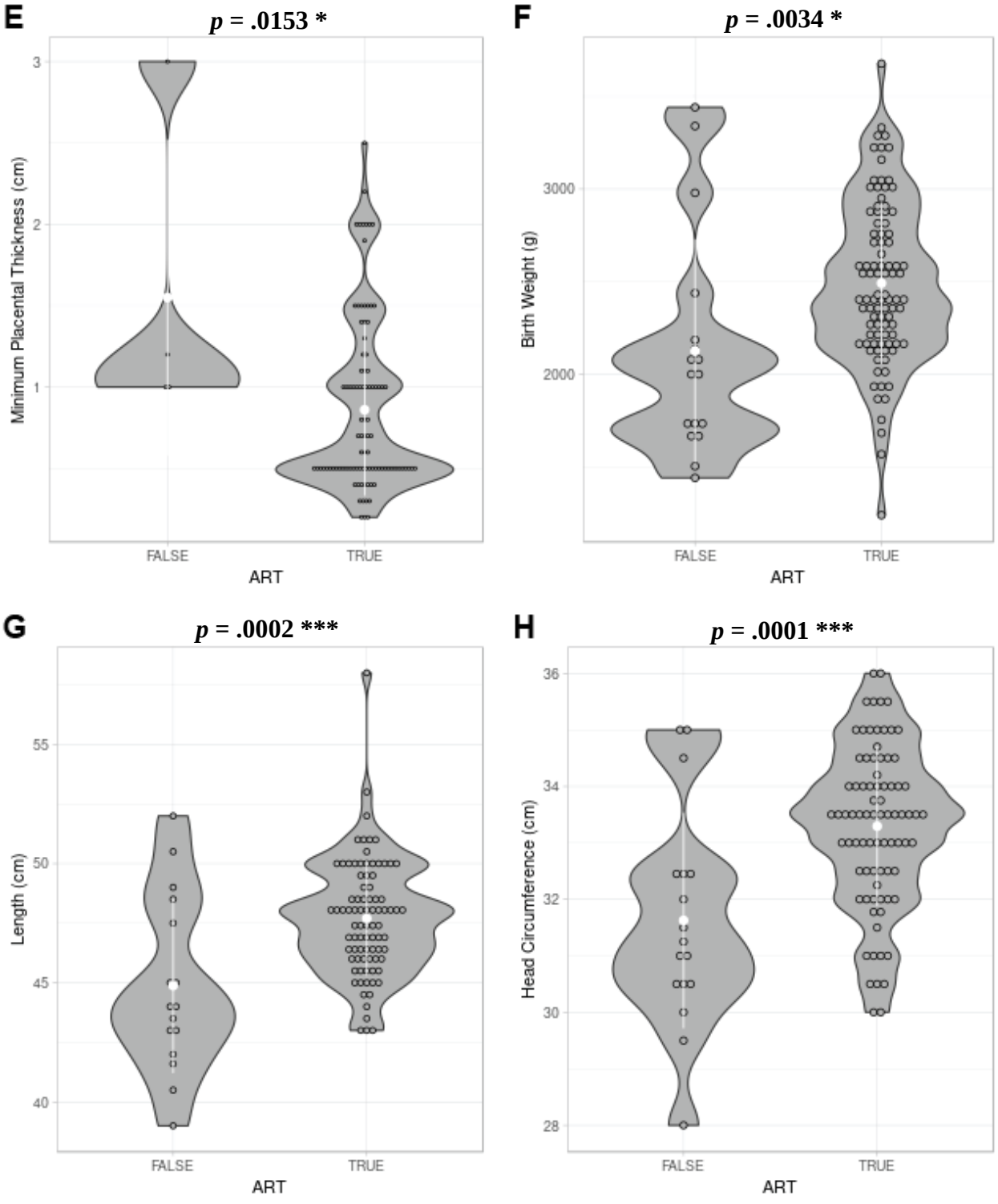


Figure 2.11. Twin birth outcomes and placental metrics by ART. Violin plots depicting distribution of birth outcomes and placental metrics by use of ART for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean

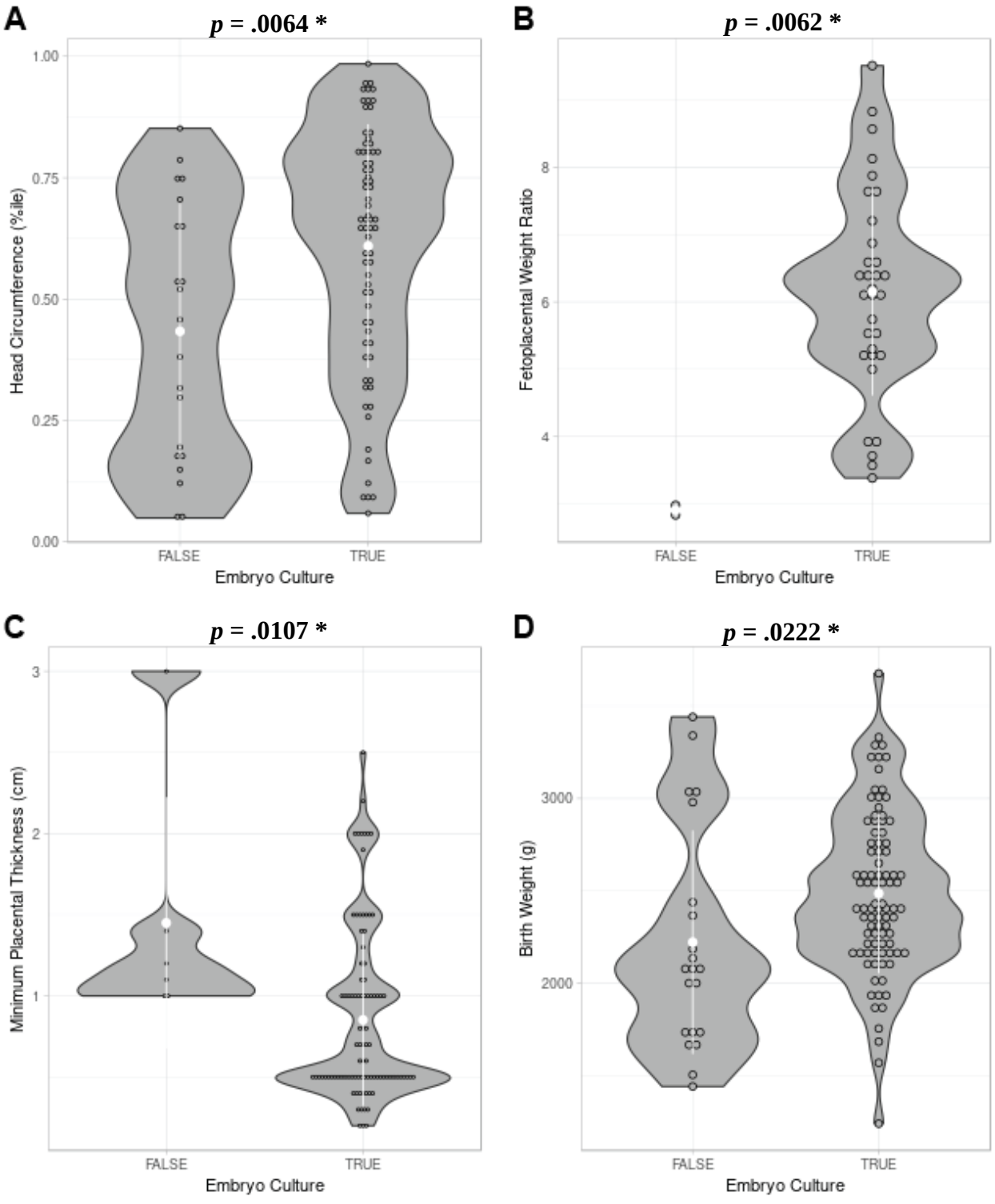
values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Body length (%ile). **C**) Head circumference (%ile). **D**) Fetoplacental weight ratio. **E**) Minimum placental thickness (cm). **F**) Unadjusted birth weight (g). **G**) Unadjusted body length (cm). **H**) Unadjusted head circumference (cm).

2.4.4 *In vitro* embryo culture

Based on whether or not the embryo was cultured *in vitro*, mean values were calculated for each observed variable and are presented below in Table 2.15. Significant differences were seen in head circumference percentile (p = .0064), FPR (p = .0062), minimum placental thickness (p = .0107), unadjusted birth weight (p = .0222), unadjusted body length (p = .0009), and unadjusted head circumference (p = .0003). Violin plots for these variables can be seen in Figure 2.12.

	No *in vitro* culture	*In vitro* culture	*p* value
n (*N* = 170)	23	147	
Gestational age (days ± SD)	250 ± 11	255 ± 11	.0737
Birth weight (percentile ± SD)	24 ± 24	31 ± 22	.2162
Body length (percentile ± SD)	39 ± 30	51 ± 24	.0539
Head circumference (percentile ± SD)	43 ± 26	61 ± 25	.0064 *
Apgar @ 1 min (± SD)	8.0 ± 1.4	8.4 ± 1.5	.3987
Apgar @ 5 min (± SD)	8.8 ± 0.5	8.9 ± 0.6	.4478
Placental weight (g ± SD)	543 ± 187	451 ± 107	.1176
F:P weight ratio (± SD)	2.9 ± 0.1	6.2 ± 1.5	.0062 *
X diameter (cm ± SD)	19.6 ± 3.8	20.8 ± 4.0	.4752
Y diameter (cm ± SD)	15.1 ± 4.0	16.4 ± 3.4	.3489
Y:X diameter ratio (± SD)	0.77 ± 0.15	0.80 ± 0.14	.5541
Min thickness (cm ± SD)	1.5 ± 0.8	0.9 ± 0.5	.0107 *
Max thickness (cm ± SD)	2.3 ± 1.3	1.8 ± 0.6	.0807
Cord diameter (cm ± SD)	1.0 ± 0.0	1.1 ± 0.3	.8152
Birth weight (g ± SD)	2223 ± 604	2487 ± 433	.0222 *
Body length (cm ± SD)	45.4 ± 3.6	47.7 ± 2.4	.0009 **
Head circumference (cm ± SD)	31.9 ± 1.9	33.3 ± 1.4	.0003 ***

Table 2.15. Twin birth outcomes and placental metrics by *in vitro* culture. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of *in vitro* embryo culturing among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.


A
p = .0064 *
Head Circumference (%ile)
1.00
0.75
0.50
0.25
0.00
FALSE
TRUE
Embryo Culture
B
p = .0062 *
Fetoplacental Weight Ratio
8
6
4
FALSE
TRUE
Embryo Culture
C
p = .0107 *
Minimum Placental Thickness (cm)
3
2
1
FALSE
TRUE
Embryo Culture
D
p = .0222 *
Birth Weight (g)
3000
2000
FALSE
TRUE
Embryo Culture

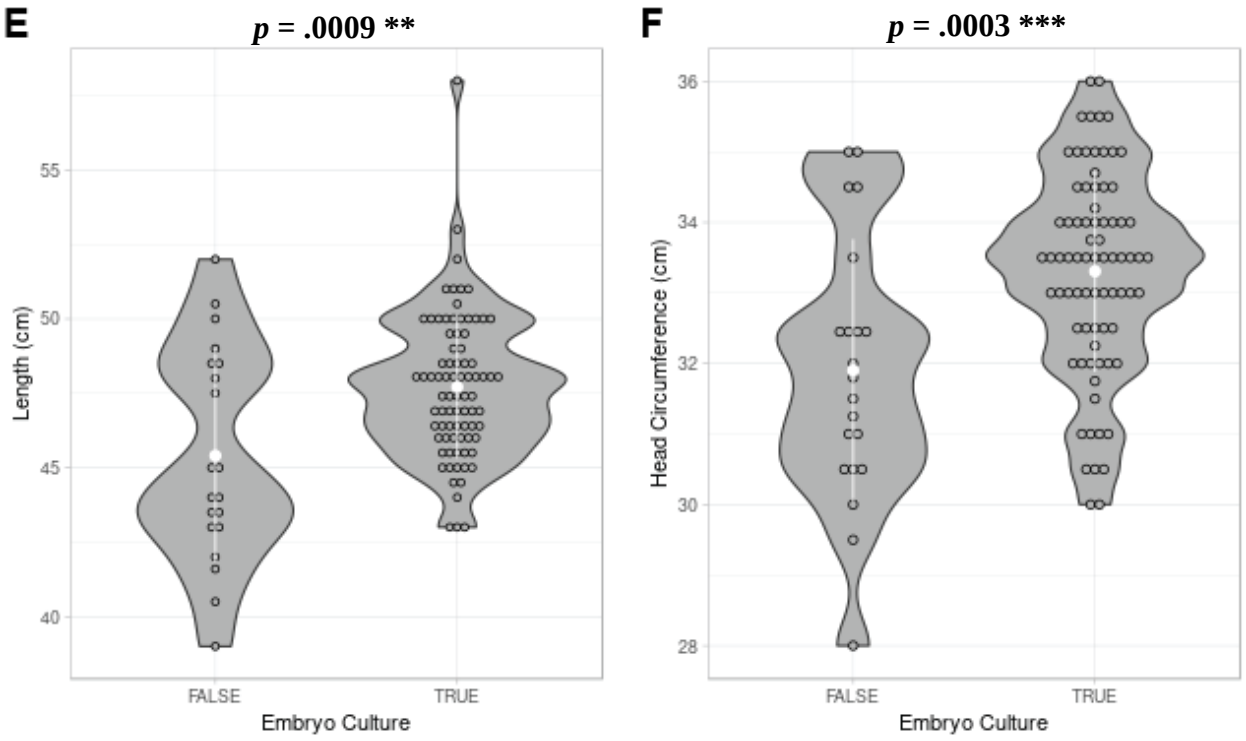


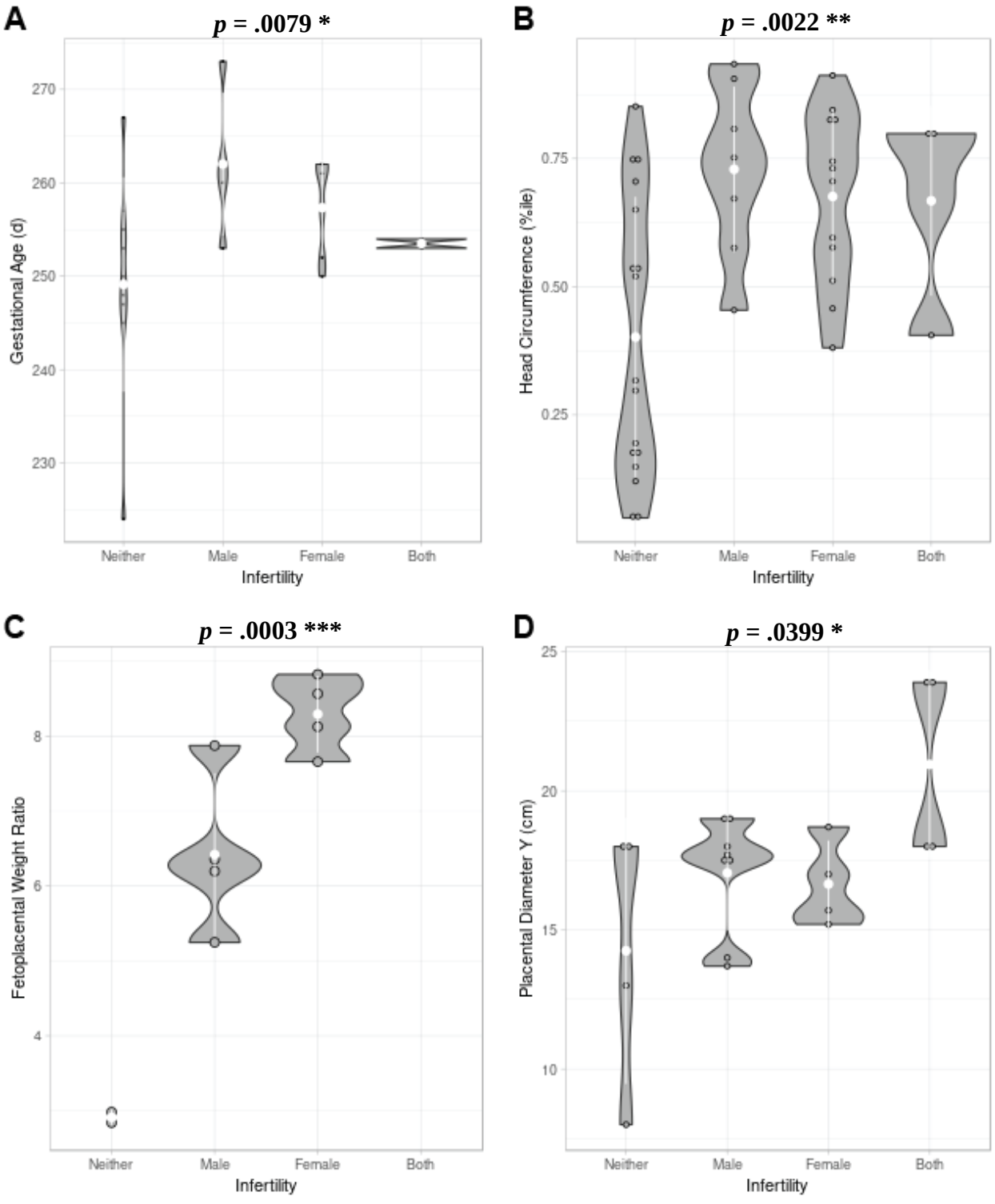
Figure 2.12. Twin birth outcomes and placental metrics by embryo culture. Violin plots depicting distribution of birth outcomes and placental metrics by use of *in vitro* embryo culture for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Head circumference (%ile). **B**) Fetoplacental weight ratio. **C**) Minimum placental thickness (cm). **D**) Unadjusted birth weight (g). **E**) Unadjusted body length (cm). **F**) Unadjusted head circumference (cm).

2.4.5 Infertility

Based on which parent was diagnosed with infertility, mean values were calculated for each observed variable and are presented below in Table 2.16. The "Neither" group is comprised of both pregnancies in which no ART was used and pregnancies in which ART was used for reasons other than infertility. The "NA" group is comprised of pregnancies in which an ART was used, but fertility status was unknown in this database and was therefor not included in the statistical comparisons. Significant differences were seen in gestational age (p = .0079), head circumference percentile (p = .0022), FPR (p = .0003), placental y diameter (p = .0399), unadjusted birth weight (p = .0170), unadjusted body length (p = .0242), and unadjusted head circumference (p = .0002). Violin plots for these variables can be seen in Figures 2.13.

	Neither	Male	Female	Both	NA	*p* value
n (*N* = 170)	19	8	12	6	125	
Gestational age (days ± SD)	249 ± 11 ^{a}	262 ± 8 ^{b}	257 ± 5 ^{ab}	254 ± 1 ^{ab}	254 ± 12	.0079 *
Birth weight (percentile ± SD)	21 ± 23	36 ± 32	38 ± 22	36 ± 34	30 ± 21	.2740
Body length (percentile ± SD)	35 ± 30	45 ± 27	60 ± 32	46 ± 33	51 ± 22	.2070
Head circumference (percentile ± SD)	40 ± 27 ^{a}	73 ± 16 ^{b}	68 ± 17 ^{b}	67 ± 19 ^{ab}	57 ± 27	.0022 **
Apgar @ 1 min (± SD)	7.8 ± 1.5	8.9 ± 0.4	7.9 ± 2.5	8.5 ± 0.6	8.4 ± 1.3	.4990
Apgar @ 5 min (± SD)	8.8 ± 0.6	9.1 ± 0.4	8.6 ± 1.0	8.5 ± 0.6	9.0 ± 0.5	.3300
Placental weight (g ± SD)	543 ± 187	459 ± 87	351 ± 18	NA ± NA	458 ± 110	.1010
F:P weight ratio (± SD)	2.9 ± 0.1 ^{a}	6.4 ± 1.1 ^{b}	8.3 ± 0.5 ^{c}	NA ± NA	5.7 ± 1.4	.0003 ***
X diameter (cm ± SD)	18.2 ± 3.4	21.2 ± 3.4	19.0 ± 2.6	22.6 ± 5.4	20.8 ± 4.1	.3400
Y diameter (cm ± SD)	14.2 ± 4.8 ^{a}	17.1 ± 2.1 ^{ab}	16.7 ± 1.6 ^{ab}	20.9 ± 3.4 ^{b}	16.1 ± 3.4	.0399 *
Y:X diameter ratio (± SD)	0.76 ± 0.13	0.82 ± 0.13	0.88 ± 0.05	0.94 ± 0.09	0.79 ± 0.14	.1640
Min thickness (cm ± SD)	1.6 ± 1.0	0.9 ± 0.6	1.0 ± 0.3	0.9 ± 0.4	0.8 ± 0.5	.3590
Max thickness (cm ± SD)	2.5 ± 1.7	2.2 ± 0.4	1.7 ± 0.4	2.1 ± 0.2	1.7 ± 0.7	.6510
Cord diameter (cm ± SD)	NA ± NA	1.1 ± 0.3	1.1 ± 0.1	1.4 ± 0.5	0.9 ± 0.2	.2360
Birth weight (g ± SD)	2128 ± 601 ^{a}	2761 ± 482 ^{ab}	2704 ± 416 ^{b}	2582 ± 484 ^{ab}	2430 ± 418	.0170 *
Body length (cm ± SD)	44.9 ± 3.7 ^{a}	48.6 ± 3.9 ^{ab}	48.6 ± 3.0 ^{b}	47.1 ± 2.4 ^{ab}	47.4 ± 2.0	.0242 *
Head circumference (cm ± SD)	31.6 ± 1.9 ^{a}	34.4 ± 0.9 ^{b}	33.8 ± 1.2 ^{b}	33.5 ± 0.7 ^{ab}	33.0 ± 1.5	.0002 ***

Table 2.16. Twin birth outcomes and placental metrics by infertility. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by source of infertility among twin births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.


A
p = .0079 *
Gestational Age (d)
270
260
250
240
230
Neither
Male
Female
Both
Infertility
B
p = .0022 **
Head Circumference (%ile)
0.75
0.50
0.25
Neither
Male
Female
Both
Infertility
C
p = .0003 ***
Fetoplacental Weight Ratio
8
6
4
Neither
Male
Female
Both
Infertility
D
p = .0399 *
Placental Diameter Y (cm)
25
20
15
10
Neither
Male
Female
Both
Infertility

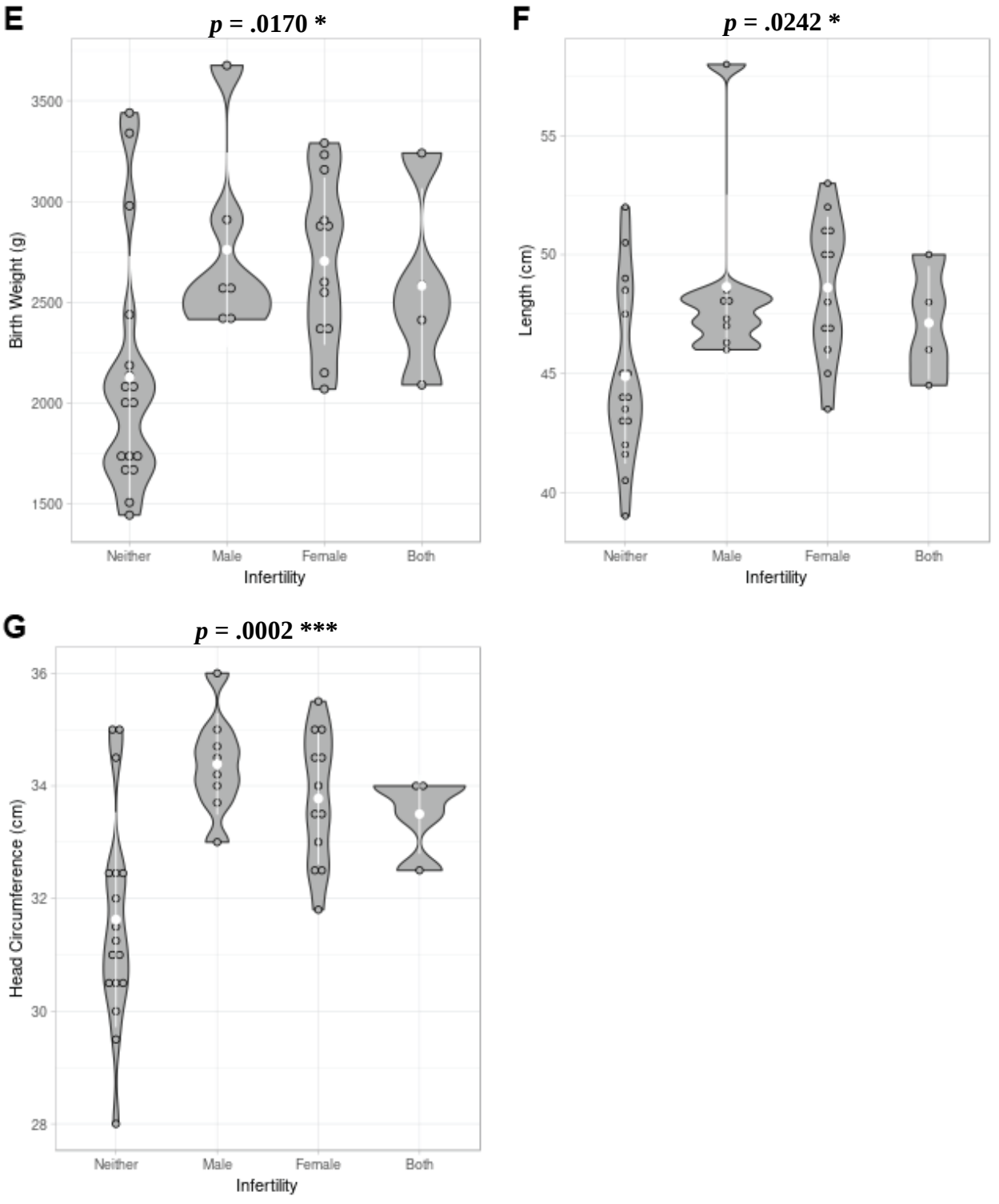


Figure 2.13. Twin birth outcomes and placental metrics by infertility. Violin plots depicting distribution of birth outcomes and placental metrics by source of infertility for each variable that was significantly different in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean

values, and white lines denote standard deviation ranges. **A**) Gestational age (d). **B**) Head circumference (%ile). **C**) Fetoplacental weight ratio. **D**) Placental y diameter (cm). **E**) Unadjusted birth weight (g). **F**) Unadjusted body length (cm). **G**) Unadjusted head circumference (cm).

2.4.6 Gravidity

Based on the mother's gravidity, mean values were calculated for each observed variable and are presented below in Table 2.17. The "NA" group is comprised of pregnancies for which the mother's gravidity was unknown in this database and was therefor not included in the statistical comparisons. A significant difference was seen in the placental y diameter (p = .0016). A violin plot for this variable can be seen in Figure 2.14.

	1	2	3	4	NA	*p* value
n (*N* = 170)	34	23	8	2	103	
Gestational age (days ± SD)	251 ± 13	258 ± 7	256 ± 3	257 ± 0	255 ± 12	.1060
Birth weight (percentile ± SD)	29 ± 22	38 ± 25	24 ± 20	39 ± 18	26 ± 22	.3270
Body length (percentile ± SD)	49 ± 24	56 ± 27	41 ± 24	88 ± 11	42 ± 26	.1020
Head circumference (percentile ± SD)	55 ± 27	55 ± 26	68 ± 27	71 ± 16	57 ± 27	.5270
Apgar @ 1 min (± SD)	8.1 ± 1.9	8.6 ± 1.0	8.9 ± 0.4	9.0 ± 0.0	8.2 ± 1.5	.3440
Apgar @ 5 min (± SD)	8.8 ± 0.9	9.0 ± 0.5	9.0 ± 0.0	9.0 ± 0.0	8.9 ± 0.4	.8750
Placental weight (g ± SD)	387 ± 55	467 ± 111	NA ± NA	348 ± 13	468 ± 118	.1870
F:P weight ratio (± SD)	8.4 ± 1.2	6.4 ± 1.1	NA ± NA	7.9 ± 0.3	5.3 ± 1.4	.0909
X diameter (cm ± SD)	18.6 ± 2.6	21.1 ± 4.1	20.2 ± 2.7	20.8 ± 2.5	21.0 ± 4.2	.4570
Y diameter (cm ± SD)	14.7 ± 1.6 [a]	16.7 ± 1.7 [ab]	18.5 ± 0.6 [b]	17.9 ± 1.2 [ab]	16.4 ± 3.8	.0016 **
Y:X diameter ratio (± SD)	0.80 ± 0.13	0.81 ± 0.15	0.92 ± 0.10	0.86 ± 0.05	0.79 ± 0.14	.4320
Min thickness (cm ± SD)	0.8 ± 0.6	1.0 ± 0.4	0.5 ± 0.1	0.9 ± 0.3	0.9 ± 0.6	.3610
Max thickness (cm ± SD)	1.7 ± 0.6	2.4 ± 0.4	2.0 ± 0.4	1.4 ± 0.1	1.8 ± 0.8	.0508
Cord diameter (cm ± SD)	0.9 ± 0.1	1.2 ± 0.3	1.3 ± 0.6	1.0 ± 0.0	1.0 ± 0.3	.0887
Birth weight (g ± SD)	2312 ± 521	2673 ± 517	2439 ± 348	2752 ± 216	2392 ± 418	.0631
Body length (cm ± SD)	46.8 ± 2.6	48.3 ± 3.2	47.0 ± 1.7	51.0 ± 1.4	46.8 ± 2.8	.0750
Head circumference (cm ± SD)	32.7 ± 1.8	33.2 ± 1.5	33.7 ± 1.3	34.0 ± 0.7	33.0 ± 1.6	.2940

Table 2.17. Twin birth outcomes and placental metrics by maternal gravidity. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by maternal gravidity among twin births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.

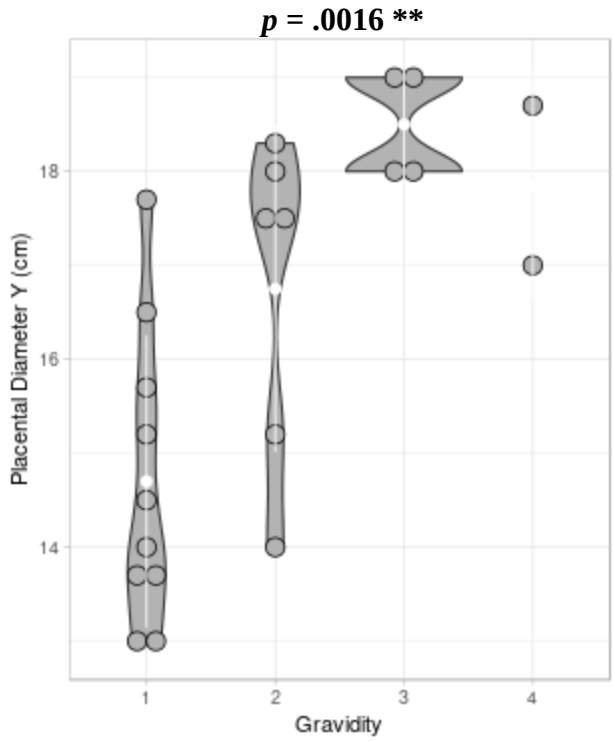


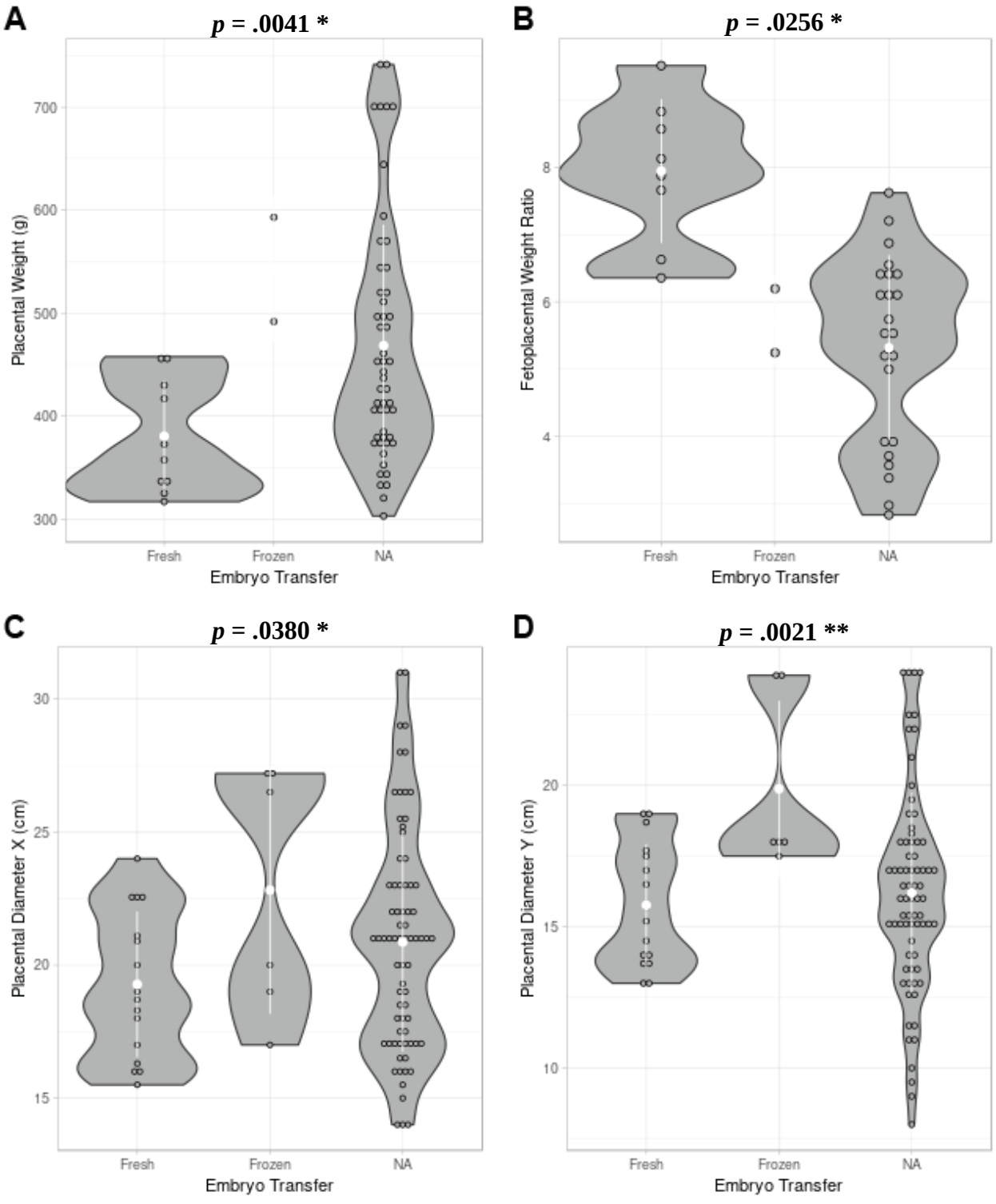
Figure 2.14. Twin birth outcomes and placental metrics by gravidity. Violin plot depicting distribution of placental y diameter (cm), the only variable that was significantly different in ANOVA, by maternal gravidity. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges.

2.4.7 Embryo transfer

Based on whether a fresh or frozen embryo was transferred, mean values were calculated for each observed variable and are presented below in Table 2.18. The "NA" group is comprised of both pregnancies in which no embryo transfer occurred (20%) and pregnancies in which an embryo transfer occurred, but fresh or frozen status was unknown in this database (80%) and was therefor not included in the statistical comparisons. Significant differences were seen in placental weight ($p = .0041$), FPR ($p = .0256$), placental x diameter ($p = .0380$), placental y diameter ($p = .0021$), maximum placental thickness ($p = .0220$), and unadjusted head circumference ($p = .0321$). Violin plots for these variables can be seen in Figure 2.15.

	Fresh	Frozen	NA	*p* value
n (*N* = 170)	38	16	116	
Gestational age (days ± SD)	256 ± 6	253 ± 16	253 ± 12	.3308
Birth weight (percentile ± SD)	31 ± 23	36 ± 22	27 ± 23	.4640
Body length (percentile ± SD)	50 ± 26	57 ± 28	46 ± 26	.3594
Head circumference (percentile ± SD)	59 ± 26	74 ± 13	51 ± 28	.0610
Apgar @ 1 min (± SD)	8.3 ± 1.7	8.7 ± 0.7	8.2 ± 1.5	.3443
Apgar @ 5 min (± SD)	8.8 ± 0.9	9.1 ± 0.4	8.9 ± 0.5	.2106
Placental weight (g ± SD)	380 ± 54	542 ± 72	468 ± 118	.0041 *
F:P weight ratio (± SD)	7.9 ± 1.1	5.7 ± 0.7	5.3 ± 1.4	.0256 *
X diameter (cm ± SD)	19.3 ± 2.7	22.8 ± 4.7	20.9 ± 4.1	.0380 *
Y diameter (cm ± SD)	15.8 ± 2.2	19.9 ± 3.1	16.2 ± 3.5	.0021 **
Y:X diameter ratio (± SD)	0.83 ± 0.11	0.89 ± 0.13	0.79 ± 0.14	.2652
Min thickness (cm ± SD)	0.8 ± 0.5	0.7 ± 0.4	0.9 ± 0.6	.6656
Max thickness (cm ± SD)	1.7 ± 0.5	2.3 ± 0.4	1.8 ± 0.8	.0220 *
Cord diameter (cm ± SD)	1.0 ± 0.2	1.3 ± 0.4	1.0 ± 0.3	.0686
Birth weight (g ± SD)	2531 ± 383	2540 ± 621	2358 ± 485	.9507
Body length (cm ± SD)	47.5 ± 2.1	49.0 ± 3.4	46.7 ± 3.0	.0761
Head circumference (cm ± SD)	33.3 ± 1.3	34.1 ± 0.8	32.6 ± 1.8	.0321 *

Table 2.18. Twin birth outcomes and placental metrics by embryo transfer. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by type of embryo transfer among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.


A
p = .0041 *
Placental Weight (g)
700
600
500
400
300
Fresh
Frozen
NA
Embryo Transfer
B
p = .0256 *
Fetoplacental Weight Ratio
8
6
4
Fresh
Frozen
NA
Embryo Transfer
C
p = .0380 *
Placental Diameter X (cm)
30
25
20
15
Fresh
Frozen
NA
Embryo Transfer
D
p = .0021 **
Placental Diameter Y (cm)
20
15
10
Fresh
Frozen
NA
Embryo Transfer

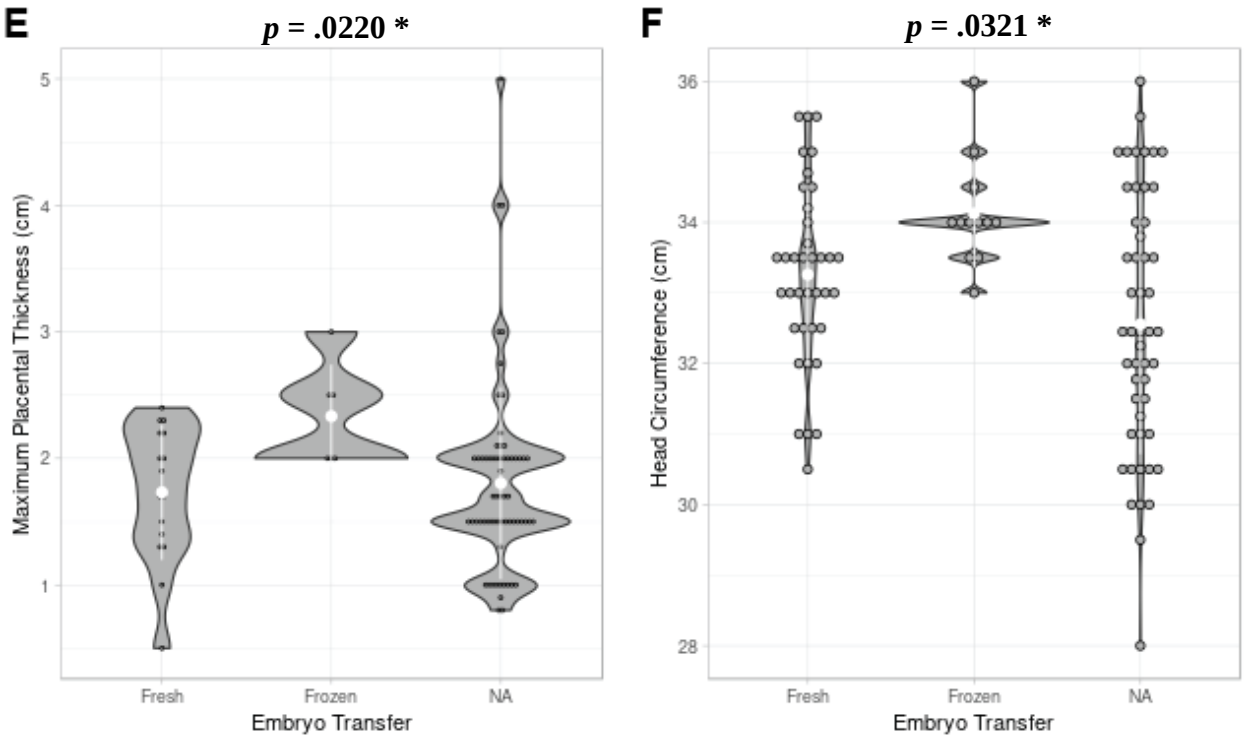


Figure 2.15. Twin birth outcomes and placental metrics by embryo transfer. Violin plots depicting distribution of birth outcomes and placental metrics by type of embryo transfer for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Placental weight (g). **B**) Fetoplacental weight ratio. **C**) Placental x diameter (cm). **D**) Placental y diameter (cm). **E**) Maximum placental thickness (cm). **F**) Unadjusted head circumference (cm).

2.4.8 Sperm donor

Based on whether or not a sperm donor was used, mean values were calculated for each observed variable and are presented below in Table 2.19. No significant differences were seen.

	No sperm donor	Sperm donor	*p* value
n (*N* = 170)	166	4	
Gestational age (days ± SD)	254 ± 11	256 ± 2	.8148
Birth weight (percentile ± SD)	30 ± 23	20 ± 9	.4056
Body length (percentile ± SD)	49 ± 26	52 ± 28	.7837
Head circumference (percentile ± SD)	56 ± 26	71 ± 26	.2812
Apgar @ 1 min (± SD)	8.3 ± 1.4	7.8 ± 2.5	.4562
Apgar @ 5 min (± SD)	8.9 ± 0.6	9.0 ± 0.0	.7464
Placental weight (g ± SD)	457 ± 114	NA ± NA	NA
F:P weight ratio (± SD)	6.0 ± 1.7	NA ± NA	NA
X diameter (cm ± SD)	20.8 ± 4.0	17.5 ± 0.7	.2526
Y diameter (cm ± SD)	16.4 ± 3.5	14.8 ± 2.5	.5052
Y:X diameter ratio (± SD)	0.80 ± 0.14	0.84 ± 0.11	.6681
Min thickness (cm ± SD)	0.9 ± 0.6	0.4 ± 0.4	.2669
Max thickness (cm ± SD)	1.8 ± 0.7	1.3 ± 0.1	.3402
Cord diameter (cm ± SD)	1.1 ± 0.3	0.8 ± 0.2	.3462
Birth weight (g ± SD)	2436 ± 486	2458 ± 140	.9280
Body length (cm ± SD)	47.2 ± 2.9	47.8 ± 1.9	.7222
Head circumference (cm ± SD)	33.0 ± 1.6	34.0 ± 1.2	.2147

Table 2.19. Twin birth outcomes and placental metrics by sperm donor. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of sperm donor among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

2.4.9 Egg donor

Based on whether or not an egg donor was used, mean values were calculated for each observed variable and are presented below in Table 2.20. Significant differences were seen in birth weight percentile (p = .0385) and body length percentile (p = .0056). Violin plots for these variables can be seen in Figure 2.16.

	No egg donor	Egg donor	*p* value
n (*N* = 170)	162	8	
Gestational age (days ± SD)	254 ± 11	254 ± 7	.9939
Birth weight (percentile ± SD)	28 ± 23	46 ± 19	.0385 *
Body length (percentile ± SD)	46 ± 26	73 ± 20	.0056 *
Head circumference (percentile ± SD)	56 ± 27	65 ± 19	.3598
Apgar @ 1 min (± SD)	8.2 ± 1.5	9.0 ± 0.0	.1943
Apgar @ 5 min (± SD)	8.9 ± 0.6	9.1 ± 0.4	.2858
Placental weight (g ± SD)	460 ± 114	348 ± 13	.1737
F:P weight ratio (± SD)	5.8 ± 1.7	7.9 ± 0.3	.0944
X diameter (cm ± SD)	20.7 ± 4.0	20.8 ± 2.5	.9777
Y diameter (cm ± SD)	16.3 ± 3.5	17.9 ± 1.2	.5381
Y:X diameter ratio (± SD)	0.80 ± 0.14	0.86 ± 0.05	.5221
Min thickness (cm ± SD)	0.9 ± 0.6	0.9 ± 0.3	.9758
Max thickness (cm ± SD)	1.8 ± 0.7	1.4 ± 0.1	.3934
Cord diameter (cm ± SD)	1.1 ± 0.3	1.0 ± 0.0	.8152
Birth weight (g ± SD)	2416 ± 481	2712 ± 348	.0910
Body length (cm ± SD)	47.1 ± 2.8	49.1 ± 2.7	.0508
Head circumference (cm ± SD)	33.0 ± 1.6	33.4 ± 1.5	.5088

Table 2.20. Twin birth outcomes and placental metrics by egg donor. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of egg donor among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

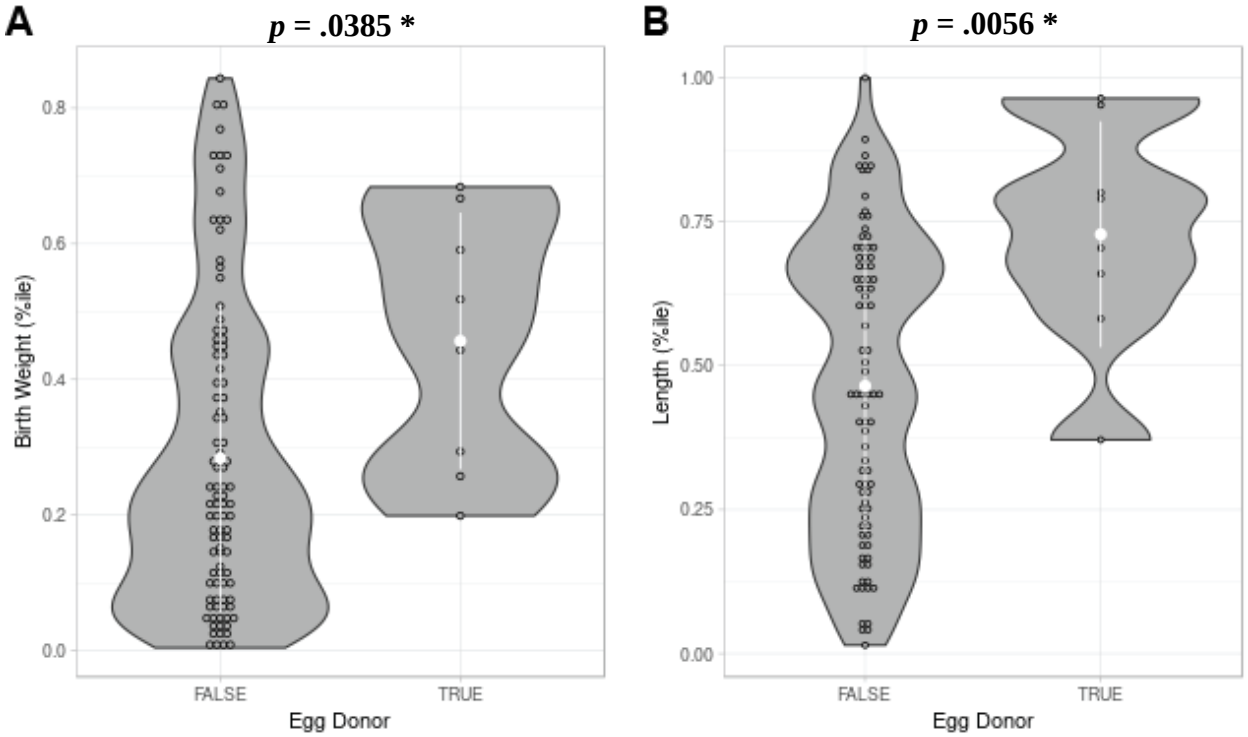


Figure 2.16. Twin birth outcomes and placental metrics by egg donor. Violin plots depicting distribution of birth outcomes and placental metrics by use of egg donor for each variable that was significantly different in Tukey HSD test. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Birth weight (%ile). **B**) Body length (%ile).

2.4.10 Surrogate

Due to the absence of any twin births following a surrogate pregnancy in this database, no comparisons were possible based on whether or not a surrogate mother was used (Table 2.21).

	No surrogate	Surrogate	*p* value
n (*N* = 170)	170	0	
Gestational age (days ± SD)	254 ± 11	NA ± NA	NA
Birth weight (percentile ± SD)	30 ± 23	NA ± NA	NA
Body length (percentile ± SD)	49 ± 26	NA ± NA	NA
Head circumference (percentile ± SD)	57 ± 26	NA ± NA	NA
Apgar @ 1 min (± SD)	8.3 ± 1.5	NA ± NA	NA
Apgar @ 5 min (± SD)	8.9 ± 0.6	NA ± NA	NA
Placental weight (g ± SD)	457 ± 114	NA ± NA	NA
F:P weight ratio (± SD)	6.0 ± 1.7	NA ± NA	NA
X diameter (cm ± SD)	20.7 ± 4.0	NA ± NA	NA
Y diameter (cm ± SD)	16.4 ± 3.4	NA ± NA	NA
Y:X diameter ratio (± SD)	0.80 ± 0.14	NA ± NA	NA
Min thickness (cm ± SD)	0.9 ± 0.6	NA ± NA	NA
Max thickness (cm ± SD)	1.8 ± 0.7	NA ± NA	NA
Cord diameter (cm ± SD)	1.1 ± 0.3	NA ± NA	NA
Birth weight (g ± SD)	2437 ± 478	NA ± NA	NA
Body length (cm ± SD)	47.3 ± 2.8	NA ± NA	NA
Head circumference (cm ± SD)	33.0 ± 1.6	NA ± NA	NA

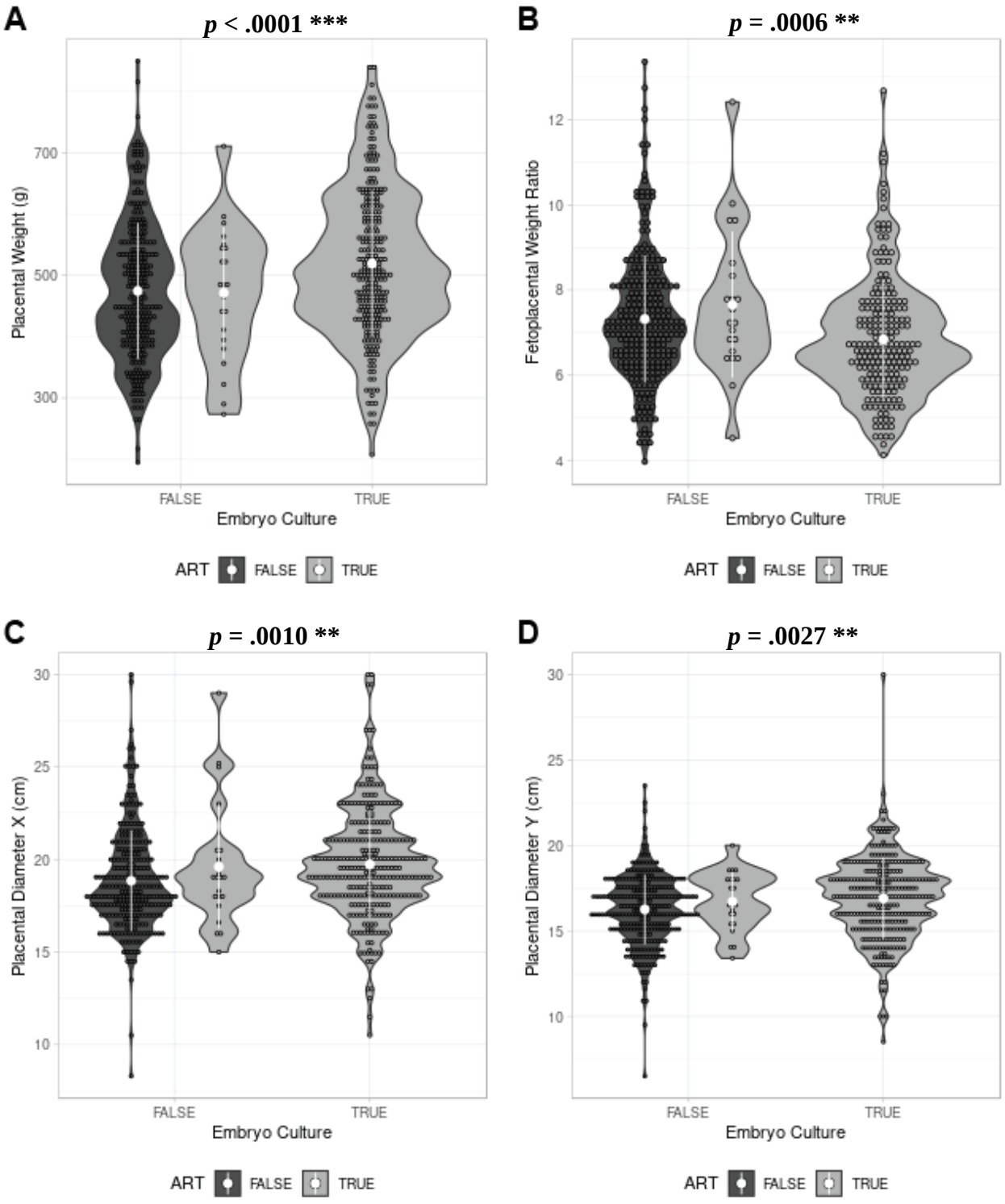
Table 2.21. Twin birth outcomes and placental metrics by surrogate. Table summarizing mean gestational age (d), birth weight (%ile), body length (%ile), head circumference (%ile), Apgar score at 1 minute, Apgar score at 5 minutes, placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), placental y:x diameter ratio, minimum placental thickness (cm), maximum placental thickness (cm), umbilical cord diameter (cm), unadjusted birth weight (g), unadjusted body length (cm), and unadjusted head circumference (cm) by use of gestational surrogate among twin births. Asterisks denote significance in Tukey HSD tests. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$.

2.5 Follow-up comparisons

Based on the use of ART and the use of *in vitro* embryo culturing, mean values were calculated for the variables seen to be significantly different most frequently throughout section 2.3 and are presented below in Table 2.22. Significant differences were seen in placental weight ($p < .0001$), FPR ($p = .0006$), placental x diameter ($p = .0010$), placental y diameter ($p = .0027$), minimum placental thickness ($p < .0001$), and maximum placental thickness ($p = .0087$). Violin plots for these variables can be seen in Figure 2.17.

	SC	ART without *in vitro* culture	ART with *in vitro* culture	*p* value
n (*N* = 700)	320	25	355	
Placental weight (g ± SD)	475 ± 111 ^{a}	471 ± 108 ^{ab}	519 ± 119 ^{b}	< .0001 ***
F:P weight ratio (± SD)	7.3 ± 1.5 ^{a}	7.7 ± 1.7 ^{a}	6.8 ± 1.4 ^{b}	.0006 **
X diameter (cm ± SD)	18.9 ± 2.7 ^{a}	19.6 ± 3.3 ^{ab}	19.7 ± 2.9 ^{b}	.0010 **
Y diameter (cm ± SD)	16.3 ± 2.0 ^{a}	16.7 ± 1.7 ^{ab}	16.9 ± 2.4 ^{b}	.0027 **
Min thickness (cm ± SD)	0.7 ± 0.4 ^{a}	0.9 ± 0.6 ^{ab}	1.1 ± 0.6 ^{b}	< .0001 ***
Max thickness (cm ± SD)	1.9 ± 0.6 ^{a}	1.9 ± 0.4 ^{ab}	2.1 ± 0.6 ^{b}	.0087 *

Table 2.22. Singleton placental metrics by ART and *in vitro* culture. Table summarizing mean placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), minimum placental thickness (cm), and maximum placental thickness (cm) by use of ART and use of *in vitro* embryo culture among singleton births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.


A
p < .0001 ***
Placental Weight (g)
700
500
300
FALSE
TRUE
Embryo Culture
ART FALSE TRUE
B
p = .0006 **
Fetoplacental Weight Ratio
12
10
8
6
4
FALSE
TRUE
Embryo Culture
ART FALSE TRUE
C
p = .0010 **
Placental Diameter X (cm)
30
25
20
15
10
FALSE
TRUE
Embryo Culture
ART FALSE TRUE
D
p = .0027 **
Placental Diameter Y (cm)
30
25
20
15
10
FALSE
TRUE
Embryo Culture
ART FALSE TRUE

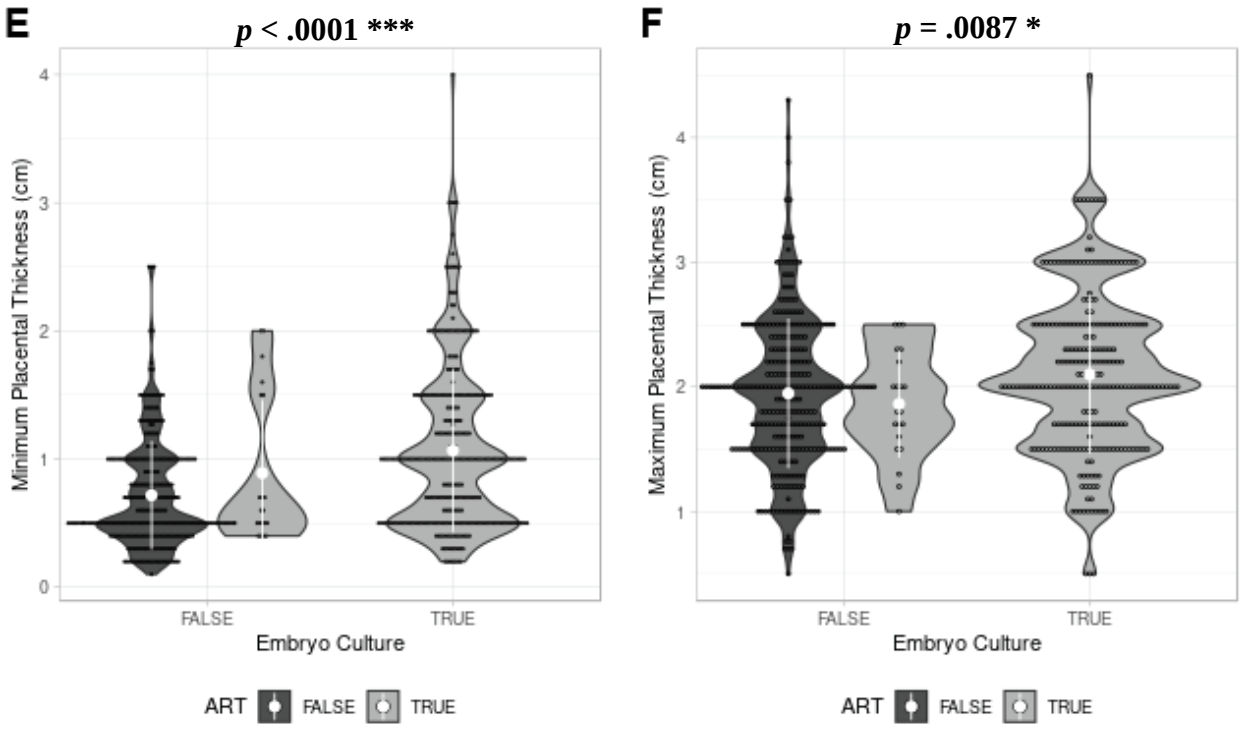
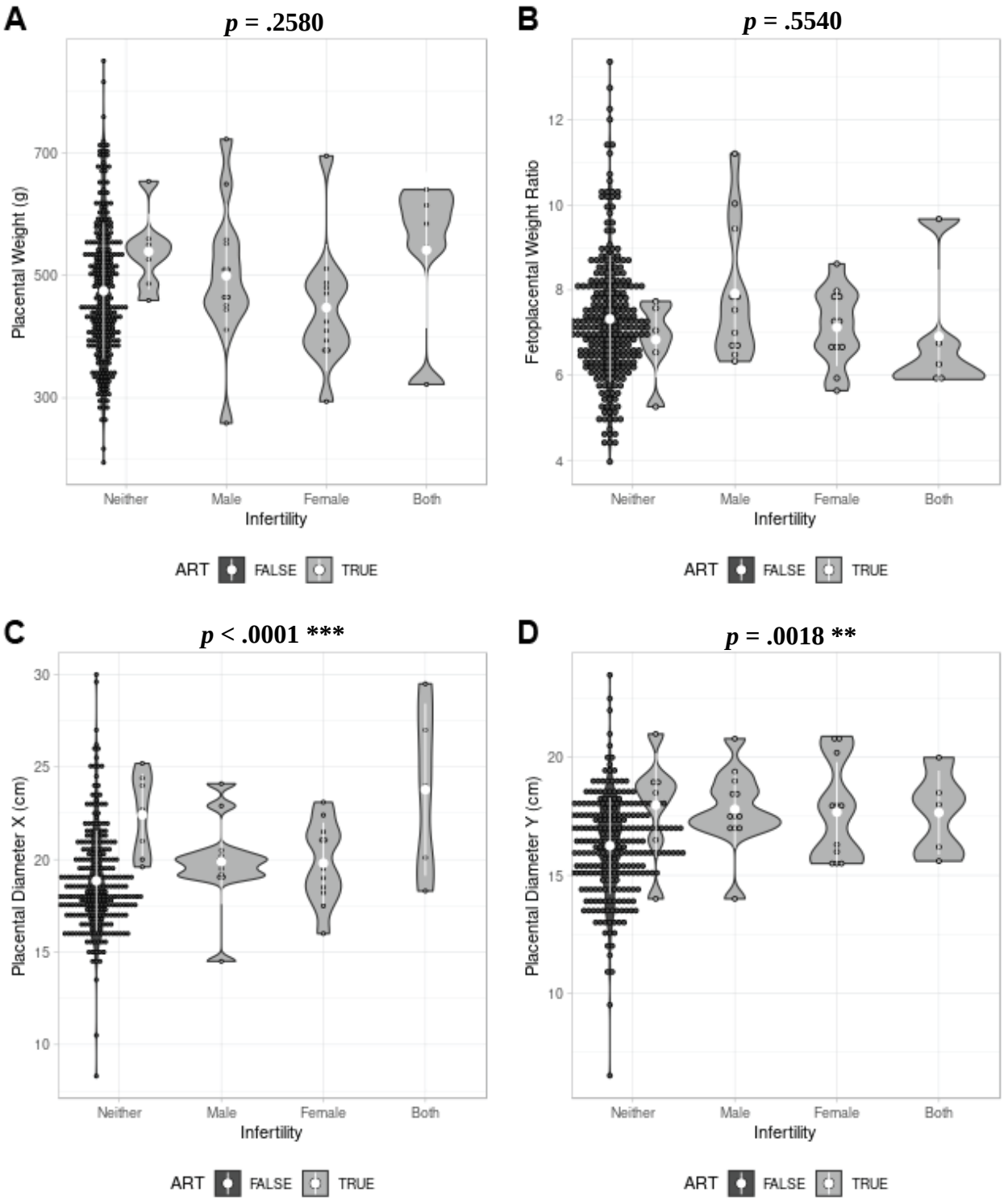


Figure 2.17. Singleton birth outcomes and placental metrics by ART and embryo culture. Violin plots depicting distribution of birth outcomes and placental metrics by use of ART and use of *in vitro* embryo culture for each variable that was significantly different in ANOVA. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Placental weight (g). **B**) Fetoplacental weight ratio. **C**) Placental x diameter (cm). **D**) Placental y diameter (cm). **E**) Minimum placental thickness (cm). **F**) Maximum placental thickness (cm).

Based on the use of ART and the source of infertility, mean values were calculated for the variables seen to be significantly different most frequently throughout section 2.3 and are presented below in Table 2.23. Significant differences were seen in placental x diameter ($p < .0001$), placental y diameter ($p = .0018$), minimum placental thickness ($p < .0001$), and maximum placental thickness ($p = .0342$). Violin plots for these variables can be seen in Figure 2.18.

	SC	ART with no infertility	ART with male infertility	ART with female infertility	ART with both infertility	*p* value
n (*N* = 365)	320	10	14	16	5	
Placental weight (g ± SD)	475 ± 111	539 ± 62	499 ± 118	447 ± 103	541 ± 128	.2580
F:P weight ratio (± SD)	7.3 ± 1.5	6.8 ± 0.9	7.9 ± 1.6	7.1 ± 0.9	6.9 ± 1.6	.5540
X diameter (cm ± SD)	18.9 ± 2.7 ^{a}	22.4 ± 2.2 ^{b}	19.9 ± 2.3 ^{ab}	19.8 ± 2.2 ^{ab}	23.8 ± 4.7 ^{b}	< .0001 ***
Y diameter (cm ± SD)	16.3 ± 2.0	18.0 ± 2.2	17.8 ± 1.7	17.7 ± 2.1	17.7 ± 1.8	.0018 **
Min thickness (cm ± SD)	0.7 ± 0.4 ^{a}	1.4 ± 0.8 ^{b}	1.2 ± 0.5 ^{bc}	1.0 ± 0.3 ^{abc}	0.7 ± 0.2 ^{ac}	< .0001 ***
Max thickness (cm ± SD)	1.9 ± 0.6	2.4 ± 0.8	2.0 ± 0.3	2.4 ± 0.7	2.2 ± 0.5	.0342 *

Table 2.23. Singleton placental metrics by ART and infertility. Table summarizing mean placental weight (g), fetoplacental weight ratio, placental x diameter (cm), placental y diameter (cm), minimum placental thickness (cm), and maximum placental thickness (cm) by use of ART and source of infertility among singleton births. Asterisks denote significance in ANOVA. * $p \leq .0500$, ** $p \leq .0029$, *** $p \leq .0003$. Superscript letters denote significance in pairwise Tukey HSD tests.


A
p = .2580
Placental Weight (g)
700
500
300
Neither
Male
Female
Both
Infertility
ART
FALSE
TRUE
B
p = .5540
Fetoplacental Weight Ratio
12
10
8
6
4
Neither
Male
Female
Both
Infertility
ART
FALSE
TRUE
C
p < .0001 ***
Placental Diameter X (cm)
30
25
20
15
10
Neither
Male
Female
Both
Infertility
ART
FALSE
TRUE
D
p = .0018 **
Placental Diameter Y (cm)
20
15
10
Neither
Male
Female
Both
Infertility
ART
FALSE
TRUE

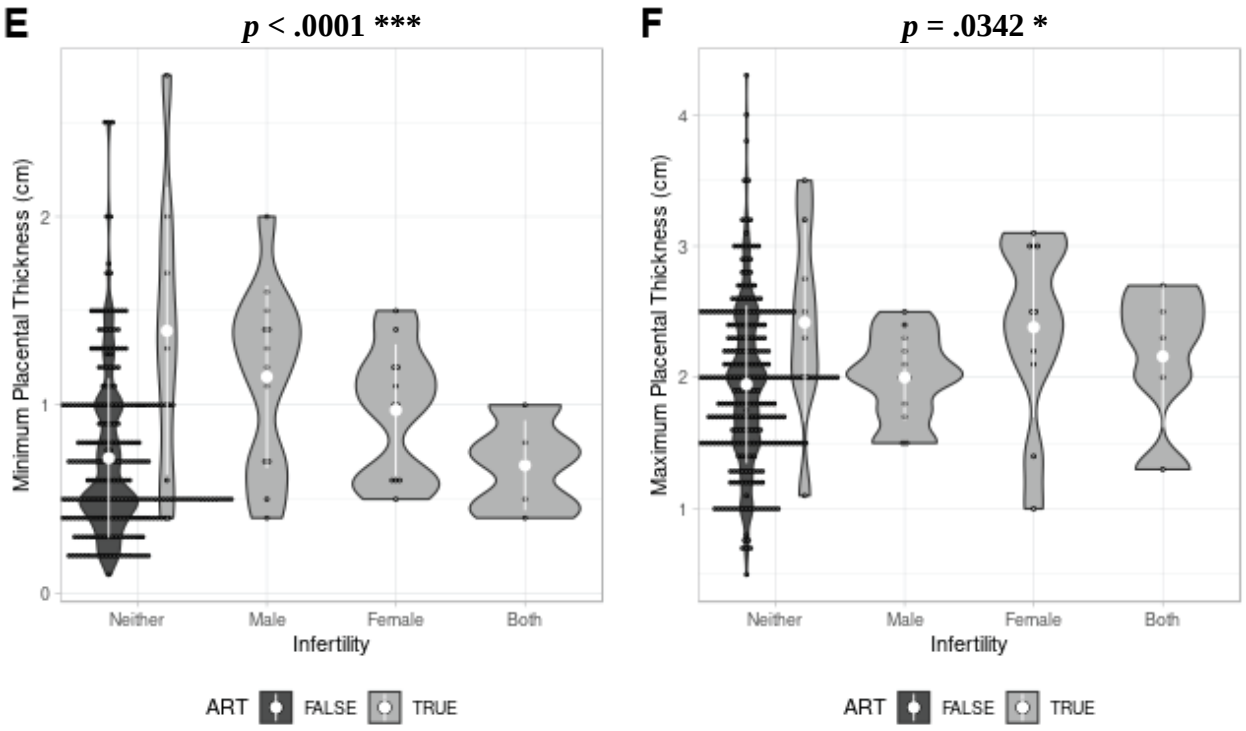


Figure 2.18. Singleton birth outcomes and placental metrics by ART and infertility. Violin plots depicting distribution of birth outcomes and placental metrics by use of ART and source of infertility for each variable that was significantly different in ANOVA. Empty circles denote individual data points, white dots denote mean values, and white lines denote standard deviation ranges. **A**) Placental weight (g). **B**) Fetoplacental weight ratio. **C**) Placental x diameter (cm). **D**) Placental y diameter (cm). **E**) Minimum placental thickness (cm). **F**) Maximum placental thickness (cm).

2.6 Discussion

In the analysis of singleton births, a total of 48 nominally significant differences were seen using the threshold of $\alpha = .050$. Because these differences were seen in all but one of the variables observed and across all comparison factors, it is likely that at least some were false positives produced by the large number of multiple comparisons. When the $\alpha = .0029$ threshold from the seventeen variable Bonferroni correction was applied, the count dropped to 20 significant differences across just four factors; ART (7), embryo culturing (7), conception (4), surrogate (1). When the threshold from the Bonferroni correction for seventeen variables across nine different grouping factors, $\alpha = .0003$, was applied, only ART, embryo culturing, and conception remained with 3, 3, and 2 significant differences, respectively. The differences

observed were largely confined to the placental metrics, especially those that remained significant after applying the stricter thresholds. The differences with *p* values below .0029 were seen in placental weight (3), minimum placental thickness (3), FPR (3), placental x diameter (3), placental y diameter (2), umbilical cord diameter (2), unadjusted body length (2), maximum placental thickness (1), and unadjusted birth weight (1). Those with *p* values of .0003 or lower were only seen in placental weight (3), minimum placental thickness (3), FPR (1), and placental x diameter (1).

Interestingly, this database did not display the lower birth weight often reported for ART births. Birth weight, body length, and head circumference were not significantly different between groups in any analysis when using the percentile values that are corrected for gestational age. Using the unadjusted values, birth weight was higher in the surrogate group, but with a sample size of just three individuals, no conclusions can be drawn. The longer ART body length seen in the conception mode, ART, and embryo culturing comparisons is more credible but given that it disappears when using the percentile values, this is more likely an artifact of the slightly longer gestational age seen for the ART groups in this database than an actual difference.

What is perhaps most surprising about the results of this analysis is the lack of a significant difference in gestational age between ART and SC newborns. Across all the comparisons performed, no group consisting of more than five individuals had a mean gestational age below 270 days or above 279 days, a typical range for healthy singleton births [99,100]. It is possible that a difference would have been detectable if not for the demographic differences between groups. Specifically, a disproportionate number of the SC cases were obtained from one particular hospital that is located in a neighboring city known to have a distinctly different ethnic composition and lower median income [101]. These factors, which have been shown to affect gestational age and weight at birth [102], may have reduced the mean value for the SC group, making a difference impossible to detect in this analysis. This result emphasizes both the challenges and the importance of obtaining well-matched controls for a study of ART outcomes.

Although gestational age and unadjusted birth weight were not significantly different as was expected, the trend of larger placentas in ART births was prevalent in this database. Given that placental weight, diameter, and thickness were consistently higher in the ART group of

multiple comparison factors, with *p* values below an α threshold that is arguably too conservative, it appears that placental size is indeed increased in ART pregnancies. Given the lack of a significant difference in birth weight, the similarly robust difference in FPR is likely due to this increased placental weight in ART births, though the previously mentioned demographic differences between groups may be affecting the mean FPR as well. What impact this increased placental size has on the health of the newborn, however, is less clear but it may reflect an altered course of fetal development as has been suggested by other studies [103]. Being larger in both surface area and total mass likely means these ART placentas contain more villous tissue for interacting with the uterine lining. This may be necessary for normal development if the placenta is less efficient at invading the endometrium or diffusing maternal blood into the fetal circulatory system. It could also suggest that the fetus is less efficient at absorbing oxygen or nutrients or has a higher than normal demand for them. Beyond the placenta's role of bringing maternal blood into close proximity to the fetal blood, more placental tissue would also allow for more production of the placenta-specific genes that are essential in fetal development [104]. Again, this may be an adaptive response to an already altered developmental track, as is suggested by the observation in mice that ART placentas continue growing later into pregnancy [33].

Seemingly contradictory to the larger placentas is the smaller umbilical cord diameter seen in the ART groups. Unless it was also less efficient in vasoconstriction, a narrower cord would restrict total blood flow to the fetus, regardless of the placenta's size and capacity for maternal blood. This would seem to suggest that the increased placental size is not allowing for greater blood flow, but is primarily an adaptation to a higher demand for placenta-specific gene production or perhaps a response to restricted blood flow through a narrower than normal umbilical cord. Though an enlarged placenta and narrower umbilical cord, especially when combined with a newborn of typical size, seems to suggest a mechanism in which the placenta is adapting to an altered developmental trajectory to preserve fetal integrity, the cause of this altered trajectory is unclear.

The different factors used for comparison in this analysis provide some insight into what is driving the differences observed. The persistence of the differences seen in the conception mode, embryo culture, and ART comparisons under very conservative α thresholds seems to

suggest that the use of ART itself, particularly those procedures involving *in vitro* culturing, are more closely associated with increased placental size than the other factors studied in this analysis. Because the comparisons based on the use of *in vitro* embryo culturing were the only ones to produce as many significant differences with similarly low *p* values as the general ART to SC comparisons, it seems likely that this aspect of ART is most responsible for the differences observed. It is unfortunate that more data on fertility status was not available in this database as the lower sample sizes for those groups make it difficult to determine whether or not infertility was driving the differences seen, though follow up comparisons using either the source of infertility or the use of *in vitro* embryo culturing as a secondary factor in addition to the use of ART do offer some insight. In comparing placental metrics based on both ART and infertility, the mean values for the pregnancies from fertile parents that underwent ART were generally more similar to those of the ART pregnancies than the SC pregnancies. In the cases of placental x diameter and minimum placental thickness, the fertile ART group was significantly different from the fertile SC group but not the infertile ART groups. This suggests that the increased placental size seen in ART pregnancies is driven more by the ART techniques themselves than the underlying infertility. In comparing IUI pregnancies, which are conceived via ART without the use of *in vitro* culturing, to SC and *in vitro* pregnancies, the mean values for the IUI group typically fell between the other two groups. In the cases of placental weight and FPR however, the IUI group was much more comparable to the SC group and was even significantly different from the *in vitro* culture group in its mean FPR. This seems to suggest that embryo culturing is a prominent factor in the increased placental size observed in ART.

Because the gamete donor and surrogate comparisons lacked the sample size to confidently detect differences, it is no surprise that few differences were seen there. The gravidity comparison, used primarily as a reference tool for showing the relative severity of any differences observed in other comparisons, was effective in showing that successive births do trend larger with slightly higher Apgar scores, but did not reach the level of significance seen in the comparisons based on ART and its components. This would suggest that ART or some aspect of it is more strongly correlated with adverse birth outcomes than a history of previous pregnancies is associated with normal, healthy birth outcomes.

In the analysis of twin births, a total of 39 significant differences across seven comparison factors were seen using the threshold of $\alpha = .050$. When the $\alpha = .0029$ threshold was applied, the count dropped to 12 significant differences across six factors; infertility (3), conception (3), ART (2), embryo culturing (2), transfer type (1), and gravidity (1). When the most stringent threshold, $\alpha = .0003$, was applied, only ART (2), infertility (1), and conception (1) remained. The differences observed in the twin analysis were less specific to the placenta than those seen in the singleton analysis. Those with *p* values below .0029 were seen in unadjusted head circumference (4), unadjusted body length (3), FPR (2), placental y diameter (2), and head circumference percentile (1). Those with *p* values of .0003 or lower were only seen in unadjusted head circumference (3), and unadjusted body length (1). These results for the twin comparisons largely corroborate the findings of the singleton comparisons with the exceptions that the increase in placental size was less significant, ART newborns appeared to be larger when comparing unadjusted values, and the infertility comparisons showed more significant differences. The less significant increase in placental size is almost certainly due to the fact that many of the twins in this database had two separate placentas that were competing for space on the endometrium, limiting their potential for growth. The seemingly larger size of ART twins compared to the SC twins is likely the difference in demographics between the two groups being exaggerated by the additional strain of a multiple pregnancy. It is also important to note the much lower sample sizes in the twin comparisons, making them less reliable for drawing firm conclusions than the singleton comparisons.

Chapter 3: Gene expression and DNAme at imprinted genes in pregnancies with low fetoplacental weight ratios

3.1 Introduction

Several studies have previously reported a lower FPR in ART births compared to SC births [30,31,34]. Though a lower ratio is quite consistent across different studies, the driving factor is not always the same. In some studies, placental weight is seen to be similar between groups, while a lower birth weight in the ART group decreases the ratio [28]. Other studies note similar birth weight between groups, but an increased placental weight in ART [31]. Some studies observe both increased placental weight and decreased birth weight in the ART group [30]. Regardless of what is driving this difference in FPR, it is of interest to know what impacts a low FPR may have on the outcome of ART pregnancies and whether or not it is associated with the purported genetic and epigenetic alterations that are sometimes seen in studies of ART children.

Because of the critical role the placenta plays in regulating fetal growth and development, it is conceivable that a change in FPR could be the cause or effect of abnormal fetal development. For example, it could suggest that the placenta is enlarged to compensate for being less efficient in its roles of nutrient transfer and gene expression [105]. It could alternatively be an indicator that the fetus is lagging in growth due to early embryonic development being disrupted by the physical and chemical stress of ART techniques. To determine if this trend of lower weight ratios in ART births is in some way associated with a more serious difference in fetal development, gene expression and DNAme data from previous studies of imprinted genes in placental villous tissue and umbilical cord blood were reanalyzed, dividing the samples not by conception mode, but on the basis of whether or not they had a low FPR.

If differences in gene expression or DNAme at the genetic regions studied are observed to be similar or more significant when comparing weight ratio groups as opposed to conception modes, it might suggest that a low FPR is indeed indicative of a more severe developmental difference between ART and SC births. If the altered gene expression observed in the studies from which this data was obtained [106] is no longer observed in the weight ratio comparison, it

would mean that a low FPR is less associated with genetic alterations than ART status itself is and may suggest that a low ratio is not necessarily an indicator of adverse fetal development.

Though the genes evaluated here were dictated by the previous studies that collected the expression and DNAme data, the rationale behind their selection still stands. The imprinted genes, *PLAGL1*, *CDKN1C*, *H19*, *IGF2*, *KCNQ1OT1*, and *PEG10* are all known to be critical for development and have been proposed to be part of an "imprinted gene network" regulated primarily by the zinc finger transcription factor PLAGL1 [107]. If the altered weight ratio between the fetus and placenta is due to an abnormal genetic or epigenetic profile, it is very likely that at least one of these genes would be involved. The repetitive element *LINE1* and its transcript *L1TD1* were also included because of their common use as a marker for global DNAme levels [108,109].

3.2 Methods

3.2.1 Study participants

Participants in this study were recruited as described in section 2.2.1. Because this was a retrospective analysis using DNAme and gene expression data collected during several different studies, the patient cohort is different for each study (Table 3.1). Differences in sample availability over time also meant that some genetic regions could not be examined for every sample in each cohort. With the exception of requiring SC pregnancies to have taken no more than one year to conceive, no specific exclusion criteria were applied so that as much of the available data as possible could be included in the FPR analysis. In dividing the cohort into "Low" and "Normal" groups for comparison, a threshold of one standard deviation below the mean FPR of SC births was used. Any births with a weight ratio more than one standard deviation below the SC mean were included in the "Low" group while all others comprised the "Normal" group.

	SC	IVF	ICSI	Total
Placental DNAme	52	29	36	117
Cord blood DNAme	46	36	34	116
Placental gene expression	31	19	16	66
Cord blood gene expression	31	10	19	60

Table 3.1. Summary of cohorts. Number of newborns conceived via SC, IVF, and ICSI in each cohort used for fetoplacental weight ratio analyses. SC, spontaneous conception; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

3.2.2 Sample preparation

Chorionic villus tissue for DNA extraction and additional villus tissue for RNA extraction were collected during placental biopsies as described in section 2.2.2. Because some tissue samples had been depleted in other studies, only two of the three RNA sites sampled, adjacent to the umbilical cord insertion site and near the edge of the chorionic plate, were used in the original gene expression study.

For each newborn in the study, approximately 10mL of umbilical cord blood was collected by hospital staff immediately after delivery. The blood samples were collected using EDTA vacuum tubes (BD Vacutainer®, NJ) and then stored at 4°C. Within 24 hours of delivery, 2.5mL of cord blood was transferred into a PAXgene™ blood RNA tube (PreAnalytix, Switzerland) and incubated at room temperature overnight before being stored at -20°C.

3.2.3 DNA extraction

After washing the chorionic villous tissue samples several times with 1x phosphate-buffered saline (PBS), 25mg of tissue was minced in a sterile petri dish using a sterile surgical blade. The tissue was then incubated overnight in lysing buffer with proteinase K in a shaking water bath at 56°C. The QIAamp™ DNA Mini Kit (Qiagen, Mississauga, ON) was then used with RNase A to avoid RNA contamination according to the manufacturer's protocol. For umbilical cord blood samples, the Qiagen Puregene Blood Core Kit C (Qiagen, Mississauga, ON) was used according to the manufacturer's instructions. After recording the DNA concentration with a NanoSpec® spectrometer (Nanovue by General Electric Inc., CT, USA), each DNA sample, from both chorionic villous tissue and umbilical cord blood, was individually

diluted and 500ng of DNA was aliquoted into a separate tube. All samples were stored at -20°C and underwent bisulphite conversion prior to pyrosequencing.

3.2.4 RNA extraction

RNA was extracted from each sample of placental chorionic villous tissue by first placing 25mg into a Lysing Matrix D tube (MP biomedicals, Santa Ana, CA) with lysing solution and β-mercaptoethanol and then homogenizing the tissue using the Bullet Blender 24 homogenizer (Next Advance, Averill Park, NY). After transferring the sample to an RNeasy™ mini spin column (Qiagen, Mississauga, ON), the Qiagen RNeasy™ Mini Kit was used to extract RNA. To verify RNA quality, samples were run on a 1.5% agarose gel (40mL TAE, 0.6g agarose, 4µL SybrSafe) at 95V for 60 minutes using 1µL of 6x orange loading dye and 2.5µL of RNA sample alongside 1µL of a low range DNA ladder. Samples were deemed to be of good quality if they produced two distinct bands for 28S and 18S ribosomal RNA with the 28S band being twice as intense as the 18S band.

Frozen umbilical cord blood samples in PAXgene™ tubes were incubated at room temperature overnight to thaw before 1mL of whole blood was taken for RNA extraction. Using the PAXgene™ Blood RNA kit (PreAnalytix, Switzerland) according to the manufacturer's instructions, RNA was extracted for each sample and then stored at -20°C. The same RNA quality check was performed as described above.

3.2.5 Preparation of cDNA library

Samples that were confirmed as being of high quality were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The cDNA library was created using either 1µg (placental chorionic villous) or 0.25µg (umbilical cord blood) of RNA for each sample and the thermal cycling profile of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and held at 4°C. All cDNA samples were briefly stored at 4°C and then diluted by a 1:50 ratio prior to RT-qPCR.

3.2.6 DNAme analysis by pyrosequencing

DNA samples underwent bisulphite conversion using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA). This technique uses sodium bisulphite to deaminate any cytosine nucleotides that are not methylated, converting them into uracil nucleotides, and leaves

methylated cytosines unchanged. The DNA sample then undergoes PCR amplification, which replaces each uracil nucleotide with a thymine. For amplification, each well received 25µL of a solution that contained HotStarTaq buffer (including 1.5mM MgCl2), 0.2 mM deoxyribonucleoside triphosphates (dNTP), 5pmol of each forward and reverse primer, and 1.0U HotStarTaq DNA polymerase (Qiagen, Mississauga, ON), as well as 2µL of bisulphite converted DNA. For negative controls, 1µL of sterilized water was used in place of DNA and for positive controls, Epitect methylated Human control DNA (Qiagen, Mississauga, ON) was used. The thermal cycling profile used for the PCR amplification was 95°C for 10 minutes, 44 cycles of (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute), 72°C for 10 minutes, and hold at 4°C. After PCR amplification, the samples were run alongside positive and negative controls on a 1% agarose gel for quality verification. Percent DNAme at each CpG site evaluated was determined using Pyro Q-CpG software, version 1.0.9 (Biotage, Foxboro, MA) on the PyroMark Q96 MD system (Biotage). Two 15µL replicates were analyzed for each DNA sample and each well contained 25µL of sterilized water, 38µL binding buffer, and 2µL of sequencing beads (all supplied by Qiagen, Mississauga, ON). For each of the four differentially methylated regions analyzed, the percent DNAme values from multiple CpG sites were averaged to obtain a single mean DNAme value for the DMR. Seven sites were analyzed in the KvDMR1 region (*KCNQ1OT1* gene promoter), including the NotI site that is often seen to be hypomethylated in BWS patients [110]. Four sites were evaluated in each of the *LINE1* and *PLAGL1* genes, and for *PEG10*, six sites were analyzed. In the case of the placental chorionic villous samples where two sites were sampled from each placenta, the mean DNAme values for each of the two tissue sites were also averaged to produce a single data point per placenta.

3.2.7 Gene expression analysis by RT-qPCR

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used with the following FAM-labeled Taqman probe and primer assays (Applied Biosystems, Foster City, CA) to evaluate the cDNA libraries: *CDKN1C* (Hs04186044_g1), *H19* (Hs00262142_g1), *IGF2* (Hs01005962_m1), *KCNQ1OT1* (Hs03456562), *L1TD1* (Hs00219458_m1), *PEG10* (Hs01122877_m1), *PLAGL1* (Hs00957794_m1). For the endogenous reference gene, *YWHAZ* (Hs01122451_m1) was used. Each sample was run in duplicate on a 96-well plate in the 7500 Real-Time PCR system (Applied Biosystems) with a thermal cycling profile of 50°C for 2

minutes, 95°C for 10 minutes, 40 cycles of (95°C for 15 seconds, 60°C for 1 minute). Each well on the plate contained either 10µL of TaqMan qPCR MasterMix (Applied Biosystems), 1µL of a target gene primer, 4µL of water, and 5µL of a cDNA sample, or 10µL TaqMan qPCR MasterMix, 1µL *YWHAZ* primer, 6.5µL water and 2.5µL of cDNA. A calibrator control was prepared by pooling equal volumes of cDNA from each of the SC pregnancy samples and relative expression (Rq) values were calculated by the ABI 7500 system software using this calibrator as a reference. Each plate also included a well in which water was used instead of cDNA (negative control) and a no reverse transcriptase well for correcting any genomic DNA contamination. The ΔΔCT method was used to analyze the results.

3.2.8 Statistical analysis

All statistical analyses were performed in the statistical program R (3.5.2) and its user interface software RStudio (1.1.456). In testing for significant differences in the clinical data, DNAme data, and gene expression data, the Tukey HSD test was used for all normally distributed numerical data. For analyzing categorical data and numerical data that did not display a normal distribution, a Kruskal-Wallis H test was performed. A minimum confidence interval of 95% ($\alpha = .050$) was used but because expression levels were being compared across four or five separate genes, a Bonferroni correction of $\alpha = .050/4 = .013$ or $\alpha = .050/5 = .010$ respectively, was applied. Analysis of the clinical information was done using the conventional $\alpha = .050$ without correction.

3.3 Results

3.3.1 Clinical information

Despite the differences in size and composition between the cohorts studied, a distinct pattern emerged in the clinical information between the "Low" and "Normal" FPR groups (Tables 3.2-3.5). For the placental DNAme cohort, cord blood DNAme cohort, placental gene expression cohort, and cord blood gene expression cohort, the mean gestational age of the "Low" FPR group was lower by 4.1 days (95% CI = 0.15, 8.13; $p = .042$), 3.6 days (95% CI = -0.41, 7.61; $p = .078$), 5.7 days (95% CI = 0.96, 10.48; $p = .019$), and 5.4 days (95% CI = -1.31, 12.06; $p = .113$), respectively. The mean birth weight of the "Low" group was lower by 156 grams

(95% CI = -66.1, 379; p = .167), 142 grams (95% CI = -84.0, 368; p = .216), 335 grams (95% CI = 38.2, 633; p = .028), and 82 grams (95% CI = -248, 413; p = .620), respectively. This trend of lower birth weight and gestational age was present in each cohort, but the difference in gestational age was only significant in the placental DNAme and placental gene expression cohorts, and the difference in birth weight was only significant in the placental gene expression cohort. Given that FPR is known to increase throughout the latter half of pregnancy, especially near the end [86], it is not surprising that a group selected for below-average FPR would also display somewhat lower gestational age and in turn, lower birth weight. The percentage of ART births was significantly different only in the placental expression cohort, with the "Low" FPR group having 29% more ART births, $\chi 2(1, N = 66) = 4.58$, p = .032. The mean maternal age and percentages of female, IUGR, LGA, and SGA births were not seen to differ significantly in any of the cohorts. These percentages were calculated using the cases in each group for which the data was known, not necessarily all the cases in each group. To determine if the differences in gestational age and birth weight between groups would influence the DNAme or expression levels of the genes being studied, Pearson correlation coefficients were calculated. None of these coefficients had an absolute value greater than r = .30, indicating that the DNAme and expression levels were not correlated with gestational age or birth weight, and are likely unaffected by the differences in group composition.

	"Normal" FPR	"Low" FPR	*p* value
n (*N* = 117)	93	24	
Mean maternal age (years ± *SD*)	33 ± 5	34 ± 5	.489
Mean gestational age (days ± *SD*)	278 ± 9	274 ± 8	.042 *
Mean birth weight (gram ± *SD*)	3374 ± 470	3217 ± 551	.167
ART (%)	54 (50/93)	62 (15/24)	.445
Female (%)	48 (45/93)	42 (10/24)	.558
IUGR (%)	1 (1/93)	4 (1/24)	.300
LGA (%)	5 (5/93)	4 (1/24)	.812
SGA (%)	11 (10/93)	17 (4/24)	.428

Table 3.2. Clinical information for placental DNAme cohort. Table summarizing mean maternal age (y), mean gestational age (d), mean birth weight (g), and percentage of ART births, female births, IUGR births, LGA births, and SGA births per conception mode in cohort of placental villous tissue DNAme study. Asterisks denote significance in Tukey HSD/ Kruskal-Wallis H test. * $p \leq .050$. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

	"Normal" FPR	"Low" FPR	*p* value
n (*N* = 116)	93	23	
Mean maternal age (years ± *SD*)	34 ± 5	34 ± 5	.679
Mean gestational age (days ± *SD*)	278 ± 9	275 ± 8	.078
Mean birth weight (gram ± *SD*)	3364 ± 470	3233 ± 548	.216
ART (%)	59 (54/92)	64 (14/22)	.595
Female (%)	50 (46/92)	50 (11/22)	.889
IUGR (%)	1 (1/92)	4 (1/22)	.282
LGA (%)	5 (5/92)	4 (1/22)	.843
SGA (%)	11 (10/92)	18 (4/22)	.384

Table 3.3. Clinical information for cord blood DNAme cohort. Table summarizing mean maternal age (y), mean gestational age (d), mean birth weight (g), and percentage of ART births, female births, IUGR births, LGA births, and SGA births per conception mode in cohort of umbilical cord blood DNAme study. Asterisks denote significance

in Tukey HSD/ Kruskal-Wallis H test. * $p \leq .050$. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

	"Normal" FPR	"Low" FPR	*p* value
n (*N* = 66)	52	14	
Mean maternal age (years ± *SD*)	35 ± 5	37 ± 6	.154
Mean gestational age (days ± *SD*)	275 ± 8	269 ± 6	.019 *
Mean birth weight (gram ± *SD*)	3377 ± 480	3024 ± 553	.028 *
ART (%)	49 (22/45)	75 (9/12)	.032 *
Female (%)	51 (23/45)	33 (4/12)	.232
IUGR (%)	0 (0/45)	8 (1/12)	.054
LGA (%)	11 (5/45)	8 (1/12)	.638
SGA (%)	9 (4/45)	17 (2/12)	.617

Table 3.4. Clinical information for placental gene expression cohort. Table summarizing mean maternal age (y), mean gestational age (d), mean birth weight (g), and percentage of ART births, female births, IUGR births, LGA births, and SGA births per conception mode in cohort of placental villous tissue gene expression study. Asterisks denote significance in Tukey HSD/ Kruskal-Wallis H test. * $p \leq .050$. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

	"Normal" FPR	"Low" FPR	*p* value
n (*N* = 60)	54	6	
Mean maternal age (years ± *SD*)	34 ± 4	32 ± 5	.481
Mean gestational age (days ± *SD*)	277 ± 7	272 ± 9	.113
Mean birth weight (gram ± *SD*)	3356 ± 394	3274 ± 260	.620
ART (%)	50 (23/46)	40 (2/5)	.443
Female (%)	48 (22/46)	80 (4/5)	.087
IUGR (%)	0 (0/46)	0 (0/5)	NA
LGA (%)	2 (1/46)	0 (0/5)	.734
SGA (%)	11 (5/46)	0 (0/5)	.431

Table 3.5. Clinical information for cord blood gene expression cohort. Table summarizing mean maternal age (y), mean gestational age (d), mean birth weight (g), and percentage of ART births, female births, IUGR births, LGA births, and SGA births per conception mode in cohort of umbilical cord blood gene expression study. Asterisks denote significance in Tukey HSD/ Kruskal-Wallis H test. * $p \leq .050$. IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

3.3.2 DNAme in placental villous tissue

The mean percent DNAme at each genomic region was determined and is presented below in Table 3.6 as percent DNAme (± SD). Aside from a nominally significant increase in LINE1 methylation, no significant differences in placental DNAme were observed between the "Low" and "Normal" FPR groups for *KCNQ1OT1* (U = 914, $p = .174$), *LINE1* (U = 776, $p = .022$), *PEG10* (U = 1159, $p = .774$), or *PLAGL1* (U = 1065, $p = .733$) (Figure 3.1).

Gene	"Normal" FPR	"Low" FPR	*p* value
KCNQ1OT1	59 ± 6.8 (n = 93)	61 ± 5.9 (n = 24)	.174
LINE1	48 ± 4.6 (n = 93)	51 ± 6.4 (n = 24)	.022 *
PEG10	50 ± 8.7 (n = 93)	49 ± 13 (n = 24)	.774
PLAGL1	46 ± 2.6 (n = 93)	47 ± 3.7 (n = 24)	.733

Table 3.6. DNAme in placenta. Table summarizing mean percent methylation (%) for *KCNQ1OT1*, *LINE1*, *PEG10*, and *PLAGL1* in placental villous tissue by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$.

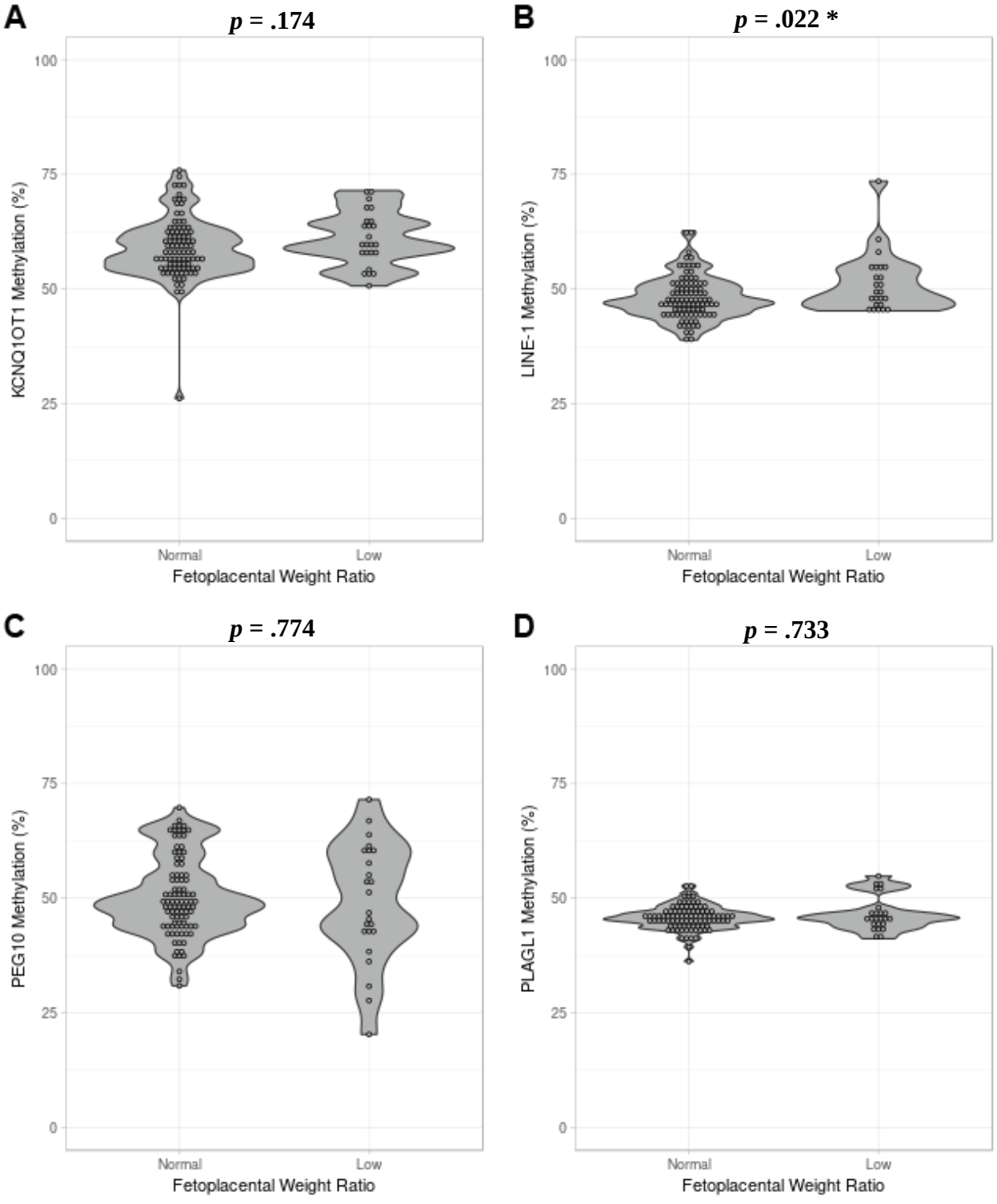


Figure 3.1. DNAme in placenta. Violin plots depicting distribution of percent methylation (%) for imprinted genes in placental villous tissue by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$. Empty circles denote individual data points. **A**) *KCNQ1OT1*. **B**) *LINE1*. **C**) *PEG10*. **D**) *PLAGL1*.

3.3.3 DNAme in umbilical cord blood

The percent DNAme at each genomic region was determined and is presented below in Table 3.7 as percent DNAme (± SD). No significant differences in umbilical cord blood DNAme were observed between the "Low" and "Normal" FPR groups for *KCNQ1OT1* (U = 789, p = .060), *LINE1* (U = 902, p = .249), *PEG10* (U = 980, p = .795), or *PLAGL1* (U = 801, p = .131) (Figure 3.2).

Gene	"Normal" FPR	"Low" FPR	p value
KCNQ1OT1	60 ± 6.8 (n = 92)	63 ± 7.1 (n = 23)	.060
LINE1	76 ± 4.5 (n = 93)	75 ± 9.7 (n = 23)	.249
PEG10	6.3 ± 11 (n = 90)	5.6 ± 11 (n = 21)	.795
PLAGL1	47 ± 4.0 (n = 92)	48 ± 3.7 (n = 22)	.131

Table 3.7. DNAme in cord blood. Table summarizing mean percent methylation (%) for *KCNQ1OT1*, *LINE1*, *PEG10*, and *PLAGL1* in umbilical cord blood by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$.

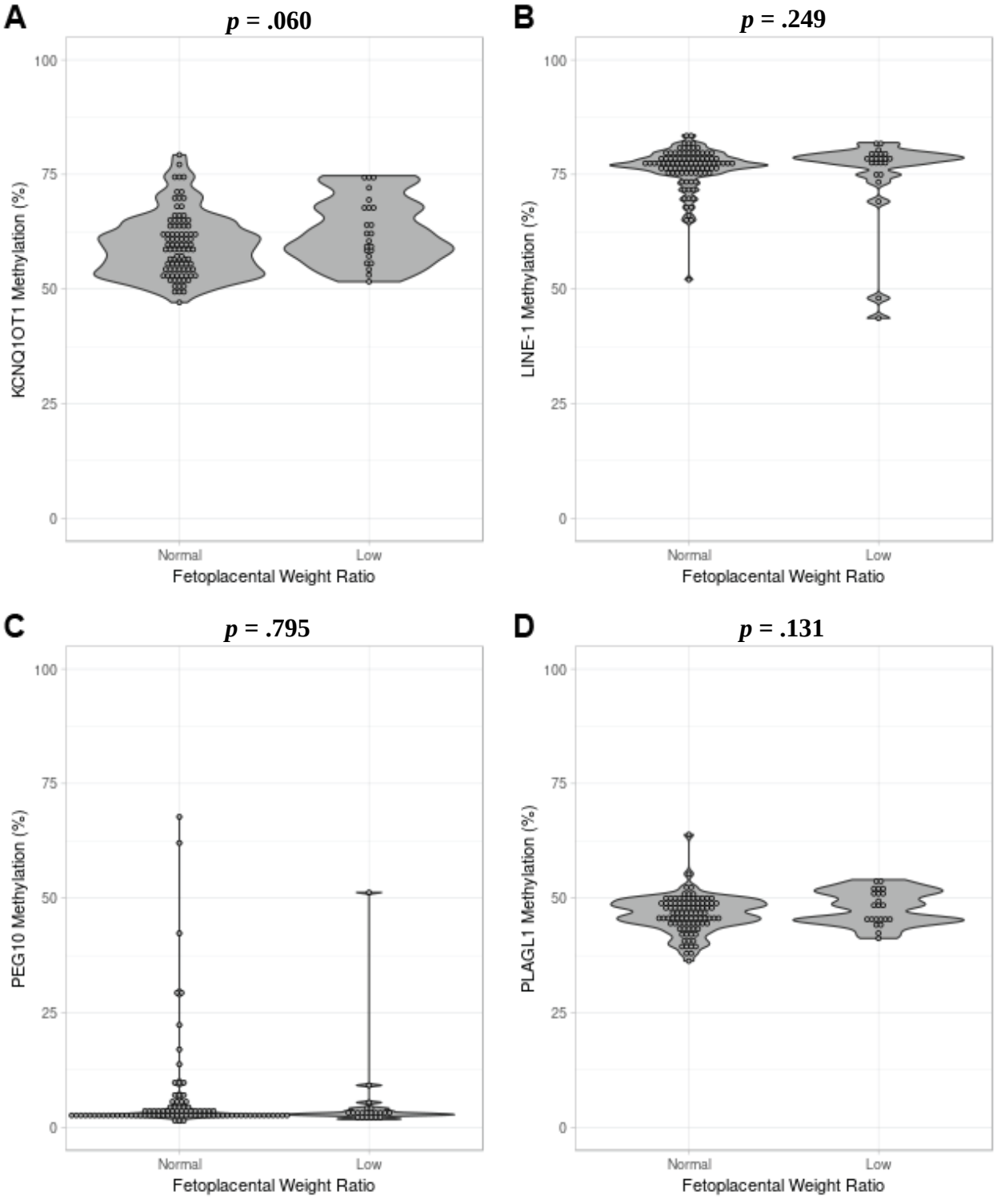


Figure 3.2. DNAme in cord blood. Violin plots depicting distribution of percent methylation (%) for imprinted genes in umbilical cord blood by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$. Empty circles denote individual data points. **A**) *KCNQ1OT1*. **B**) *LINE1*. **C**) *PEG10*. **D**) *PLAGL1*.

3.3.4 Gene expression in placental villous tissue

The relative expression level of each gene was determined by RT-qPCR and is presented below in Table 3.8 as Rq (± SD). No significant differences in placental expression were observed between the "Low" and "Normal" FPR groups for *KCNQ1OT1* (U = 467, p = .108), *L1TD1* (U = 169, p = .328), *PEG10* (U = 209, p = .666), or *PLAGL1* (U = 209, p = .739) (Figure 3.3).

Gene	"Normal" FPR	"Low" FPR	p value
KCNQ1OT1	1.5 ± 2.0 (n = 52)	1.8 ± 3.1 (n = 14)	.108
L1TD1	1.2 ± 1.0 (n = 35)	1.2 ± 0.51 (n = 12)	.328
PEG10	1.1 ± 0.61 (n = 46)	1.4 ± 1.2 (n = 10)	.666
PLAGL1	1.5 ± 1.3 (n = 45)	1.9 ± 2.0 (n = 10)	.739

Table 3.8. Gene expression in placenta. Table summarizing mean relative expression (Rq) for *KCNQ1OT1*, *L1TD1*, *PEG10*, and *PLAGL1* in placental villous tissue by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$.

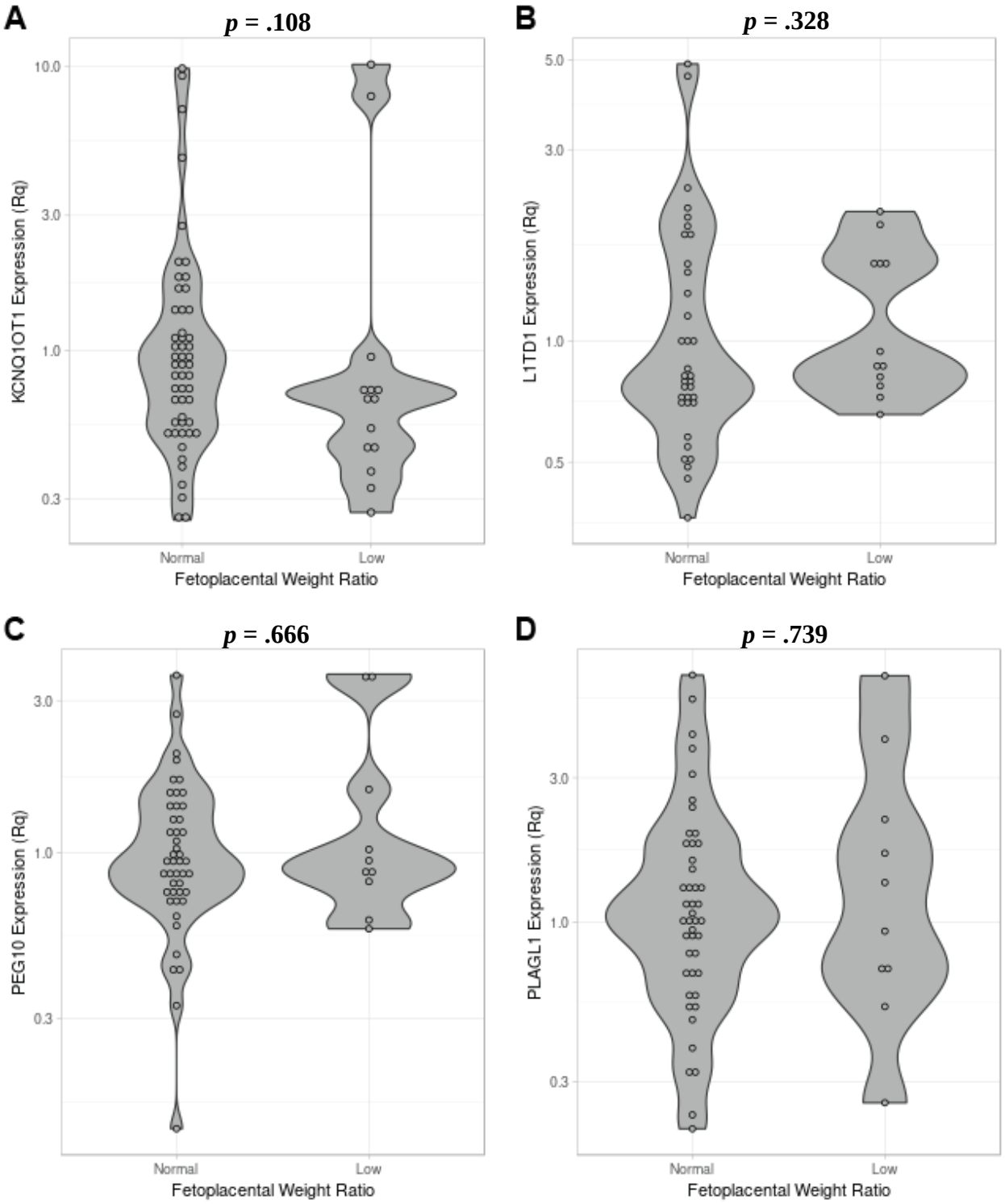


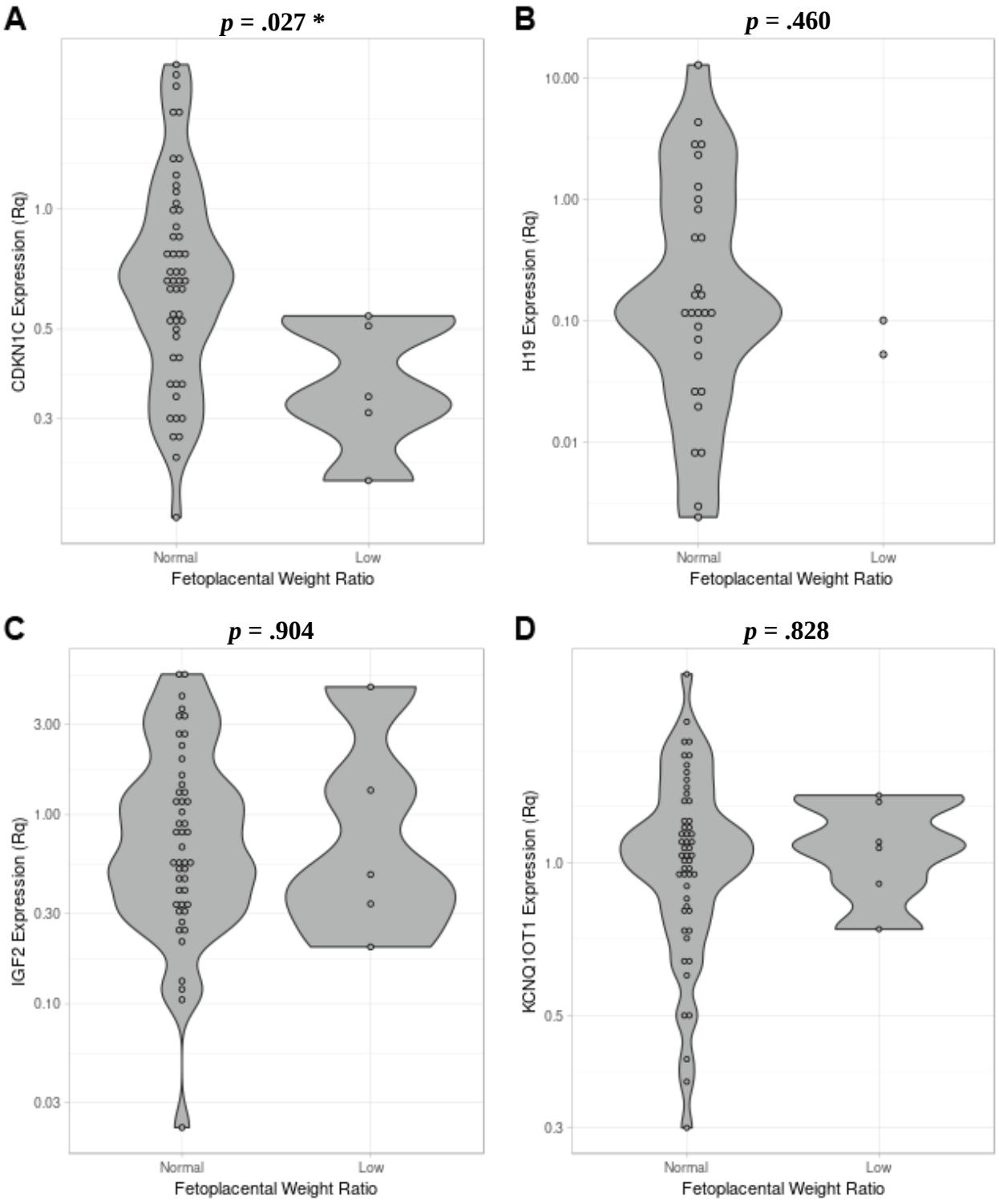
Figure 3.3. Gene expression in placenta. Violin plots depicting distribution of relative expression (Rq) for imprinted genes in placental villous tissue by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .013$. Empty circles denote individual data points. **A**) *KCNQ1OT1*. **B**) *L1TD1*. **C**) *PEG10*. **D**) *PLAGL1*.

3.3.5 Gene expression in umbilical cord blood

The relative expression level of each gene was determined by RT-qPCR and is presented below in Table 3.9 as Rq (± SD). Aside from a nominally significant decrease in *CDKN1C* expression, no significant differences in umbilical cord blood expression were observed between the "Low" and "Normal" FPR groups for *CDKN1C* (U = 201, p = .027), *H19* (U = 38, p = .460), *IGF2* (U = 122, p = .904), *KCNQ1OT1* (U = 147, p = .828), or *PLAGL1* (U = 154, p = .969) (Figure 3.4).

Gene	"Normal" FPR	"Low" FPR	p value
CDKN1C	0.78 ± 0.50 (n = 50)	0.38 ± 0.14 (n = 5)	.027 *
H19	1.1 ± 2.5 (n = 28)	0.077 ± 0.034 (n = 2)	.460
IGF2	1.2 ± 1.4 (n = 47)	1.4 ± 1.9 (n = 5)	.904
KCNQ1OT1	1.1 ± 0.40 (n = 52)	1.1 ± 0.24 (n = 6)	.828
PLAGL1	0.79 ± 0.25 (n = 52)	0.79 ± 0.25 (n = 6)	.969

Table 3.9. Gene expression in cord blood. Table summarizing mean relative expression (Rq) for *CDKN1C*, *H19*, *IGF2*, *KCNQ1OT1*, and *PLAGL1* in umbilical cord blood by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .010$.


A
p = .027 *
CDKN1C Expression (Rq)
1.0
0.5
0.3
Normal
Low
Fetoplacental Weight Ratio
B
p = .460
H19 Expression (Rq)
10.00
1.00
0.10
0.01
Normal
Low
Fetoplacental Weight Ratio
C
p = .904
IGF2 Expression (Rq)
3.00
1.00
0.30
0.10
0.03
Normal
Low
Fetoplacental Weight Ratio
D
p = .828
KCNQ1OT1 Expression (Rq)
1.0
0.5
0.3
Normal
Low
Fetoplacental Weight Ratio

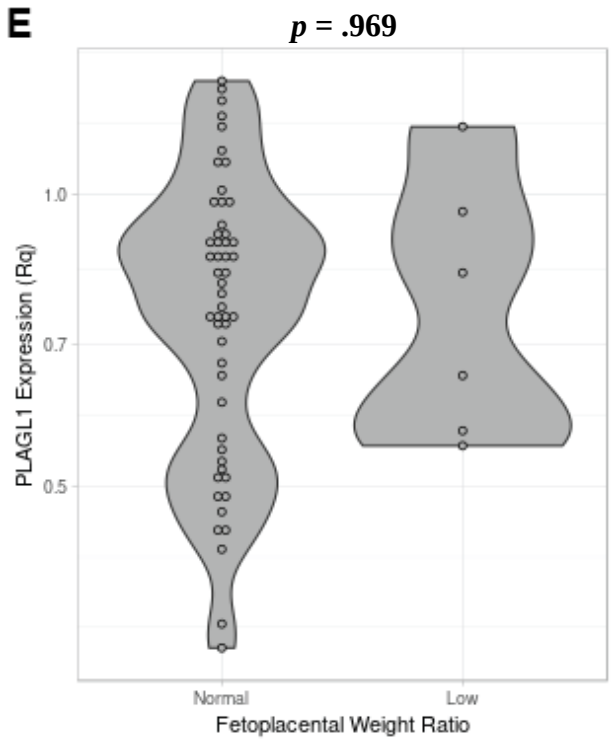


Figure 3.4. Gene expression in cord blood. Violin plots depicting distribution of relative expression (Rq) for imprinted genes in umbilical cord blood by "Normal" or "Low" fetoplacental weight ratio. Asterisks denote significance in Tukey HSD tests. * $p \leq .050$, ** $p \leq .010$. Empty circles denote individual data points. **A**) *CDKN1C*. **B**) *H19*. **C**) *IGF2*. **D**) *KCNQ1OT1*. **E**) *PLAGL1*.

3.4 Discussion

Across all four cohorts, no significant differences between the "Low" and "Normal" FPR groups were seen in gene expression or DNAme using the Bonferroni-corrected thresholds of $\alpha = .013$ and .010. There were however two nominally significant differences that met the conventional threshold of $\alpha = .050$; *LINE1* DNAme in placenta ($p = .022$), and *CDKN1C* expression in cord blood ($p = .027$). Little can be said of the difference in *CDKN1C* expression due to the low sample size in that cohort, but the minor increase in *LINE1* DNAme could potentially indicate disrupted DNAme regulation in low FPR pregnancies as *LINE1* is somewhat reflective of global DNAme levels [108,109]. In the original studies from which this data was obtained, the expression of three genes differed in umbilical cord blood [106]. In the IVF group, expression of *IGF2* was higher and *KCNQ1OT1* was lower when compared to the SC group. In both the IVF and ICSI groups, *PLAGL1* was expressed at lower levels than the SC group. This

also corresponded to an increase in cord blood DNAme at *PLAGL1* in the IVF group during the original studies. In the current study, however, none of those differences remained even nominally significant. Overall, no trace of the previously reported associations remains in the new weight ratio comparison. This would seem to suggest that the previously observed differences in gene expression are not directly associated with lower FPRs.

Though the results of this study do not support a direct connection between a low FPR and purported changes in ART gene expression, they do not exclude the possibility that an atypical weight ratio is associated with other risk factors. Past studies have found adverse birth outcomes such as IUGR and even stillbirth to occur at greater rates among children born with an abnormally low FPR [111]. It is still entirely possible that both a low weight ratio and altered DNAme and gene expression are side effects of infertility or the ART techniques used to treat it. It may just be that the two effects occur independently of one another and neither one affects every ART birth. Overall, this study does not indicate that a low FPR in itself causes or even indicates genetic or epigenetic abnormalities.

The greatest weakness of this study is most likely its reliance on previous studies for data. Because the samples were not originally selected to represent the "Low" and "Normal" FPR groups, the sample sizes were unbalanced between groups and extremely small in some cases. This is especially true for the cord blood gene expression cohort where sample sizes of 2, 5, and 6 made drawing any firm conclusions impossible. Using data from previous studies also restricted gene selection and meant that the cohorts were not the same for each individual analysis. Another important caveat to keep in mind for this study is the lack of a standard threshold for determining "Low" and "Normal" weight ratios. While this study was limited by the data that was available, perhaps an analysis with a more stringent cutoff of 1.5 or 2.0 standard deviations below the mean weight ratio would produce different results.

Chapter 4: Gene expression in umbilical cord blood from pregnancies with adverse birth outcomes

4.1 Introduction

Numerous studies have compared gene expression levels in children conceived via ART to those of children conceived spontaneously [73,76,77,79-82,112-114]. One tissue that is often used in these studies is umbilical cord blood. Along with placental tissue, cord blood can be easily obtained from a large number of patients due to its simple and non-invasive method of collection. Unlike the extraembryonic placental tissue, however, umbilical cord blood is derived from the embryonic cell lineage, making it a better representation of the newborn's own genetic composition. Using cord blood for gene expression studies does share a significant drawback with the placenta, however, that being its high degree of variability in cell type composition [115].

Due to different tissue types, different sample collection methods, variation in cell composition, and several other factors, gene expression studies in ART versus SC newborns have often produced conflicting results, and there are few if any genes that demonstrate a consistent change in ART births. Expression levels of some imprinted genes, such as *H19* and *IGF2*, are frequently seen to be altered though not always in the same direction and sometimes not at all [71,73,79-82,114]. One plausible factor in explaining the inconsistent observations is that gene expression is only being altered in a subset of ART births that is not equally represented across different studies. The increased number of preterm, multiple, and growth-restricted births seen in the ART population may be one such subset.

To determine if these adverse birth outcomes may be contributing to the variability in gene expression, a cohort of newborns exhibiting preterm, twin, IUGR, SGA, and LGA births was selected for comparing umbilical cord blood gene expression in ART and SC births. Due to the atypical nature of a cohort selected to contain adverse outcomes, variation in imprinted gene expression is to be expected in both the ART and SC groups relative to the more stable endogenous control genes used for reference. However, if the differences in expression display any trends or patterns that are unique to the ART group, it may shed light onto what role these

adverse birth outcomes are playing in the altered gene expression sometimes observed in ART births.

The four genes selected for this study, *H19*, *HYMAI*, *PHLDA2*, and *PLAGL1*, were chosen for their status as imprinted genes that have previously been associated with altered expression in ART births [79-81]. Because of their essential roles in regulating fetal development, they are each associated with at least one imprinting disorder [59,116,117], developmental disorders that appear to occur more frequently in ART births [35-39]. They also give representation to both PEGs (*HYMAI*, *PLAGL1*) and MEGs (*H19*, *PHLDA2*).

4.2 Methods

4.2.1 Study participants

Participants in this study were recruited as described in section 2.2.1.

4.2.2 Sample preparation

Umbilical cord blood samples were collected and prepared as described in section 3.2.2.

4.2.3 RNA extraction

RNA was extracted as described in section 3.2.4.

4.2.4 Preparation of cDNA library

Samples that were confirmed as being of high quality were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The cDNA library was created by using 1 µg of RNA for each sample and the thermal cycling profile of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and hold at 4°C. Each sample was reverse transcribed in duplicate with a third replicate containing no reverse transcriptase to be used in controlling for genomic DNA contamination. The cDNA samples were stored overnight at 4°C and diluted by a 1:6 ratio prior to RT-qPCR.

4.2.5 Gene expression analysis by RT-qPCR

RT-qPCR was used with the following FAM-labeled Taqman probe and primer assays (Integrated DNA Technologies, Coralville, IA) to evaluate the cDNA libraries: *H19*

(Hs.PT.58.2694336.g), *HYMAI* (Hs.PT.58.25312231), *PHLDA2* (Hs.PT.58.598689.g), *PLAGL1* (Hs.PT.58.26731331). For the endogenous reference genes, *UBC* (Hs.PT.39a.22214853) and *YWHAZ* (Hs.PT.58.23092985) were used. Each sample was run in duplicate on a 96-well plate in the 7500 Real-Time PCR system (Applied Biosystems) with a thermal cycling profile of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of (95°C for 15 seconds, 60°C for 1 minute). Each well on the plate contained either 10µL of TaqMan qPCR MasterMix (Applied Biosystems), 1µL of a target gene primer, and 9µL of a cDNA sample, or 10µL TaqMan qPCR MasterMix, 1µL of an endogenous reference gene primer, 7µL water and 2µL of cDNA. A calibrator control was prepared by pooling equal volumes of cDNA from each of the SC pregnancy samples and relative expression (Rq) values were calculated by the ABI 7500 system software using this calibrator as a reference. Each plate also included a well in which water was used instead of cDNA (negative control). The ΔΔCT method was used to analyze the results.

4.2.6 Statistical analysis

All statistical analyses were performed in the statistical program R (3.5.2) and its user interface software RStudio (1.1.456). In testing for significant differences in the clinical data and relative expression of each gene, the Kruskal-Wallis H test was used for all categorical data and any numerical data that did not display a normal distribution. For comparing the normally distributed data, an ANOVA test was performed. Each Kruskal-Wallis H test and ANOVA test that showed a significant difference was followed up with pairwise comparisons using the Mann-Whitney U test or Tukey HSD test, respectively. A minimum confidence interval of 95% (α = .050) was used, but because expression levels were being compared across four separate genes, a Bonferroni correction of α = .050/4 = .013 was used. Analysis of the clinical information was done using the conventional α = .050 without correction.

4.3 Results

4.3.1 Clinical information

Maternal age was seen to be significantly different between conception modes with the IVF group having a higher mean maternal age than the SC group by 5.95 years (95% CI = 1.22, 10.7; p = .009) (Table 4.1). The number of twins and SGA births was also significantly different

between the IVF and SC groups with the IVF group having 42.6% more twins, $\chi 2(1, N = 41) = 9.25$, $p = .002$ and 35.0% fewer SGA births, $\chi 2(1, N = 41) = 5.44$, $p = .020$. Given the similarly high percentage of twins in the ICSI group, as well as the high maternal ages and low SGA percentages in both the ICSI and IUI groups when compared to the IVF group, it is likely that these groups would have also been significantly different than the SC group if their sample sizes were larger. When comparing the percentage of IUGR births across groups, each of the ART groups had significantly fewer than the SC group: the IVF group had 51.9% fewer, $\chi 2(1, N = 41) = 11.6$, $p < .001$, the ICSI group had 61.9% fewer, $\chi 2(1, N = 25) = 4.95$, $p = .026$, and the IUI group had 61.9% fewer, $\chi 2(1, N = 24) = 3.88$, $p = .049$. The mean gestational age, mean birth weight, and percentage of females and LGA births did not differ significantly between conception modes. The percentages reported for female, IUGR, LGA, and SGA births were calculated using the cases in each group for which the data was known, not necessarily all the cases in each group. To determine if the difference in maternal age between groups would affect the expression levels of the genes being studied, Pearson correlation coefficients were calculated. None of these coefficients exceeded an absolute value of r = .27 (*PLAGL1*), indicating that the expression levels were not correlated with maternal age, and are likely unaffected by the difference in group composition.

	SC	IUI	IVF	ICSI	*p* values
n (*N* = 48)	21	3	20	4	
Mean Maternal Age (years ± *SD*)	30 ± 6 [a]	36 ± 1 [ab]	36 ± 5 [b]	34 ± 3 [ab]	.018 *
Mean Gestational Age (days ± *SD*)	247 ± 10	254 ± 4	248 ± 13	252 ± 4	.582
Mean Birth Weight (gram ± *SD*)	2078 ± 570	2542 ± 581	2359 ± 454	2268 ± 104	.119
Female (%)	33 (7/21)	33 (1/3)	40 (8/20)	75 (3/4)	.486
Twin (%)	52 [a] (11/21)	67 [ab] (2/3)	95 [b] (19/20)	100 [ab] (4/4)	.011 *
IUGR (%)	62 [a] (13/21)	0 [b] (0/3)	10 [b] (2/20)	0 [b] (0/4)	.001 ***
LGA (%)	5 (1/20)	0 (0/3)	0 (0/20)	0 (0/4)	.717
SGA (%)	50 [a] (10/20)	0 [ab] (0/3)	15 [b] (3/20)	0 [ab] (0/4)	.028 *

Table 4.1. Clinical information for adverse outcome births. Table summarizing mean maternal age (y), mean gestational age (d), mean birth weight (g), and percentage of female births, twin births, IUGR births, LGA births, and SGA births per conception mode. Asterisks denote significance in ANOVA/Kruskal-Wallis H test. * $p \leq .050$, ** $p \leq .010$, *** $p \leq .001$. Superscript letters denote significance in pairwise Tukey HSD/Mann-Whitney U tests. SC, spontaneous conception; IUI, intrauterine insemination; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; IUGR, intrauterine growth restriction; LGA, large for gestational age; SGA, small for gestational age.

4.3.2 Gene expression of umbilical cord blood

The relative expression of each gene was determined for each mode of conception and is presented below in Table 4.2 as Rq (± *SD*). In each of the four genes analyzed, no significant differences in umbilical cord blood expression were observed between the different modes of conception. For the expression of *H19*, *HYMAI*, *PHLDA2*, and *PLAGL1*, the ANOVA results were $F(3,44) = 0.91$, $p = .446$, $F(3,44) = 1.02$, $p = .391$, $F(3,44) = 0.13$, $p = .940$, $F(3,44) = 0.67$, $p = .575$, respectively (Figure 4.1).

	SC	IUI	IVF	ICSI	*p* values
n (*N* = 48)	21	3	20	4	
H19	20 ± 57	0.45 ± 0.37	1.4 ± 2.8	0.82 ± 1.2	.446
HYMAI	1.1 ± 0.47	1.4 ± 0.51	1.4 ± 0.71	1.1 ± 0.33	.391
PHLDA2	1.5 ± 1.5	1.2 ± 0.61	1.3 ± 0.89	1.7 ± 1.4	.940
PLAGL1	1.1 ± 0.48	1.3 ± 0.20	1.1 ± 0.26	1.3 ± 0.38	.575

Table 4.2. Gene expression in adverse outcome births. Table summarizing mean relative expression (Rq) for *H19*, *HYMAI*, *PHLDA2*, and *PLAGL1* in umbilical cord blood by mode of conception. Asterisks denote significance in ANOVA. * $p \leq .050$, ** $p \leq .013$.

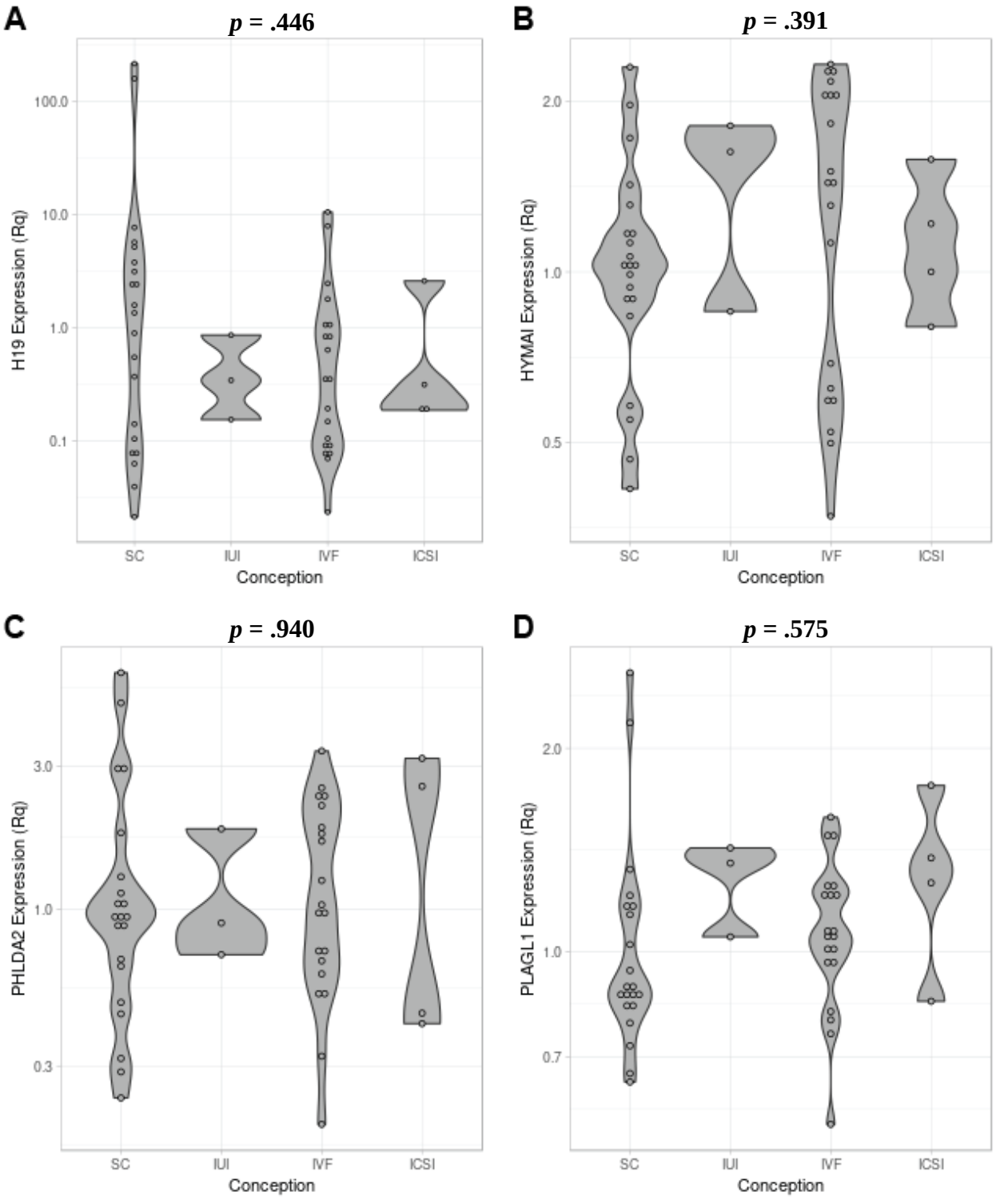


Figure 4.1. Gene expression in adverse outcome births. Violin plots depicting distribution of relative expression (Rq) for imprinted genes in umbilical cord blood by mode of conception. Asterisks denote significance in ANOVA. * $p \leq .050$, ** $p \leq .013$. Empty circles denote individual data points. **A**) *H19*. **B**) *HYMAI*. **C**) *PHLDA2*. **D**) *PLAGL1*.

4.4 Discussion

The fact that there were no significant differences in the expression of the genes studied would seem to suggest that the use of ART alone is not the primary driver in the expression differences sometimes noted in studies of ART births. With the exception of a pair of SC twins who had much greater expression of *H19*, all four groups displayed similar distributions of expression levels of each gene. Though not represented by the mean values, and not statistically significant, *PLAGL1* expression levels did seem to be trending higher in the ART groups, with the more invasive techniques having higher values. This is what would be expected if the technical factors of ART, namely *in vitro* culturing and cell membrane puncture, were having a cumulative effect. Complicating this observation, however, is the fact that the percentage of twins in each group also increases with the same pattern. Given *PLGAL1*'s role as a cell growth suppressor, an increase in its expression would most likely be associated with decreased fetal growth. As both ART and twin births are generally associated with lower birth weight, it is impossible to determine from this study if this slight increase in *PLAGL1* expression is a response to the ART techniques or the strains of a twin pregnancy. This emphasizes the importance of controlling for multiple births in studies of ART pregnancies, as they are known to occur at a greater frequency after the use of ART.

If the minor increase in *PLAGL1* expression is an effect of the increased percentage of twins in the ART groups, this study would suggest that pregnancy complications such as twinning, preterm birth, and growth restriction are more associated with the differences in gene expression sometimes seen in ART children than the ART techniques themselves. Given the greater rate of occurrence of these complications in ART pregnancies [2-5,27-29], it is understandable how they could give rise to an apparent change in ART gene expression, though most studies today filter for such factors, studying only term singletons with normal growth patterns. There does exist the possibility of pregnancy complications going undetected or unreported in ART gene expression studies and causing the purported differences, however, the question of what causes these complications to disproportionately affect ART pregnancies would still remain.

In addition to the low sample sizes, particularly in the IUI and ICSI groups, there are other factors that make it difficult to draw any firm conclusions from this study. Perhaps the

most significant is the variability in group composition. Because the number of samples from pregnancies with adverse birth outcomes was rather limited, the factors of multiple, preterm, IUGR, SGA, and LGA birth were not equally represented across the groups. A larger study that could separate these factors into different analyses would be better suited to determine what role each one plays in influencing gene expression. Differences in the sex ratio and mean maternal age between groups is another element of the study that makes it difficult to draw conclusions as sex and maternal age have both been reported to affect genetic regulation in human oocytes, embryos, and newborns [118-121].

The genes studied here, as well as most other genes that have been reported to be altered in ART births, are not always seen to be altered to the same degree or even in the same direction. As a gene that is frequently studied in the context of ART, *H19* has been reported to be increased [80,81], decreased [79], and unaltered [76,114] in ART multiple times. Though there is currently no consensus, there does appear to be more reports of the expression of these and other imprinted genes being unaltered rather than altered in ART children [76,79,82,114,122]. One study noted that the expression levels of four imprinted genes (*IGF2, H19, KCNQ1OT1, CDKN1C*) were not significantly different in ART newborns of appropriate birth weight when compared to SC newborns of appropriate birth weight [76]. When they looked at growth-restricted births, however, they saw *H19* to be decreased in both groups, *CDKN1C* decreased in the ART group, and *KCNQ1OT1* increased in the SC group. In other words, gene expression was more variable in growth-restricted births, but remained unaltered in births of normal growth and weight, regardless of ART or SC status. This suggests that the ART techniques themselves are not driving gene expression changes. It is possible however, that the use of ART allows for a greater occurrence of altered expression due to more stochastic drivers such as epigenetic or genetic abnormalities inherited from infertile parents.

Chapter 5: Conclusion

5.1 Major Findings and Implications

In Chapter 2, a database of 889 live births was analyzed to compare birth outcomes in newborns conceived via ART to those conceived spontaneously. This analysis did not detect the lower birth weight and gestational age that is often reported for ART newborns. It did, however, identify a significantly larger placenta and consequently, lower FPR in ART births. This increase in placental size was observed in measurements of weight, vertical thickness, and horizontal width on two perpendicular axes. A significantly smaller umbilical cord diameter was also observed. These differences were seen across multiple comparisons using different factors of pregnancy and ART procedures to divide the samples. The factors that produced the most significant differences were whether or not ART was used, whether or not the embryo was cultured *in vitro*, and which specific mode of conception resulted in the pregnancy. Because these factors display a large degree of overlap with each other, as well as the fertility concerns that necessitate the use of ART, the differences seen do not directly point to a cause for the enlarged placentas.

Some insight is available, however, where the IUI group, cases of infertility that did not use *in vitro* culturing, aligns more with fertile SC group than the other infertile groups that did use *in vitro* embryo culturing. This seems to suggest that the embryo culturing may be a major driver of the placental size differences seen between ART and SC newborns. Additionally, the small number of cases in which fertile couples used ART for reasons other than infertility showed placenta sizes more similar to the other ART groups than the fertile control group. This seems to be the strongest piece of evidence in this analysis as to what is driving the observed differences in placental size, and it points to the use of ART. Though it resulted in few significant differences, the comparison of cases divided by the source of infertility did seem to suggest that infertility may also be affecting birth outcomes, albeit to a lesser degree. It is conceivable that due to its heterogeneity in causes and mechanisms, infertility does not produce a uniform effect that consistently alters birth outcomes in all pregnancies or in any particular direction and is, therefore, less detectable in studies such as this one. Along this line of

reasoning, it would seem that the consistent alterations in ART births, such as a larger placenta, are due to the ART procedures while the more stochastic effects, such as altered DNAme and gene expression, are the result of the underlying infertility. Intuitively, it makes sense that the negative effects of infertility would manifest themselves in the genes and epigenetic marks of suboptimal gametes in a manner as variable and random as infertility itself. Similarly, if an established clinical technique such as *in vitro* fertilization is responsible for adverse birth outcomes, it would be expected that the effects would mirror the procedure's prevalence and consistency as was seen with placental metrics in this thesis.

In Chapter 3, previous gene expression and DNAme data from umbilical cord blood and placental villous tissue were compared between births with "Low" and "Normal" FPRs. In each comparison, no significant difference was seen, despite the original studies finding significant differences when dividing the groups by whether or not they were conceived using ART. These results suggest then that the lower FPR that is often seen in ART is not directly associated with altered DNAme and expression. As mentioned in Chapter 2, the lower weight ratio appears to primarily be a result of the larger placental size, which itself seems to be an adaptive response to otherwise altered development rather than the cause of it. This is largely speculative, however, and certainly needs to be studied further. The lack of association between a low FPR and altered DNAme or gene expression does support the notion proposed above that genetic and epigenetic abnormalities occur in some percentage of ART births as a result of the underlying infertility while enlarged placentas are a prevalent adaptive response to the stress an embryo endures during *in vitro* culturing.

In Chapter 4, imprinted gene expression in umbilical cord blood was compared between ART and SC newborns that were selected for their adverse birth outcome status. Though numerous limitations to the study make it difficult to draw firm conclusions, no significant differences were detected for any of the genes that were studied. As is expected for a cohort selected for adverse outcomes, expression levels were seen to vary but to no greater degree or with any distinct trends in the ART group. This would seem to suggest that among pregnancies complicated by twinning, preterm birth, undergrowth, and overgrowth, ART newborns are not at any more of an increased risk of genetic abnormalities than are SC newborns, at least in the genes studied here.

Overall, the clinical impact of these findings is rather minimal. They seem to suggest that any genetic or epigenetic abnormalities seen in ART births are due more to the underlying infertility than the techniques being performed in the clinic. If that is the case, it can only be addressed by pre-implantation genetic screening, something that is already offered and used somewhat regularly when there is reason for concern. The indication that culturing embryos *in vitro* may be the primary driver of enlarged placentas has the greatest potential for clinical impact. It would suggest that clinics should try to minimize the amount of time an embryo spends in culture and regularly re-evaluate which culture medium supplier they choose to purchase from, as new formulas may offer better outcomes for both their embryo transfers and their patients' pregnancies. Neither of these ideas is new to the clinical IVF community, however, and both are quite common in practice already.

5.2 Limitations

Throughout these studies, there were numerous limitations to keep in mind while considering the results. One weakness that was present in all three chapters was the demographic heterogeneity of the cohorts. As mentioned in Chapter 2, ethnic and socioeconomic differences can affect birth outcome as well as gene expression. Because the cases used in these studies were collected from several different cities across western Canada, with different conception modes disproportionately representing different cities, it is entirely possible that the results were affected by the demographic composition of the cohorts. Correcting for these differences was not possible as data for ethnicity was only collected in the last few years of sample collection and data for socioeconomic status was never collected. Similar to ethnic background, other data points such as embryo transfer type and origin of infertility were added to the patient questionnaire several years into sample collection, resulting in the large number of NA cases seen throughout Chapter 2.

Another limitation that affected every study was the small number of samples available for certain analyses. Because Chapter 2 used the entire database, most of the singleton comparisons had sufficient group sizes, but the limited data for the use of surrogates and gamete donors meant a much lower power for detecting any differences that may have been present. The studies in Chapters 3 and 4 were even more affected by the issue of small sample sizes. The gene

expression study in pregnancies with adverse outcomes was limited in size and scope by design as it was intended to be a pilot test for a larger study. The analyses performed in Chapter 3 were restricted in sample size due to their reliance on previous studies for data. This also meant that the genetic regions to be evaluated were limited to the genes already studied. These studies could certainly benefit from being redesigned with the explicit intention of comparing FPR groups. The use of RT-qPCR was another limiting factor in the size of the studies in Chapters 3 and 4 as it becomes very costly when analyzing many genes. It would be of great interest to expand these studies using a more genome-wide approach such as RNA sequencing and an 850K methylation array as there is no reason to suspect that all genes would show the same results seen in the few genes studied here.

The use of cases that are fundamentally different as a single group was perhaps the most concerning limitation to the studies presented here, particularly in Chapter 4. In the adverse outcome gene expression study, there was a large degree of variability in what defined the cases as having adverse outcomes. Some cases displayed a large for gestational age birth weight while others were small for their gestational age. Some cases were born preterm but of average weight, while others were term births that were growth-restricted. Additionally, some samples came from twins, while others were from singletons. This issue of trying to compare singletons to twins was also present in the cohorts of Chapter 3.

5.3 Future Directions

Given the findings of these studies and their many limitations, there is ample opportunity to build upon and improve this line of investigation. As mentioned above, the studies discussed in Chapters 3 and 4 could be significantly improved if they were redesigned. The cohorts for the FPR study could be reselected to better balance the "Low" and "Normal" group sizes. Additionally, a high weight ratio group could be added, or cases could be divided into quantiles after ranking by FPR to provide a deeper understanding of how this metric is associated with gene expression or birth outcome beyond just the low ratio seen in ART. It would also be of interest to build on the study by expanding the cohort to all births, testing for associations with other pregnancy complications and genetic or epigenetic profiles. This may shed some light on

what exactly is causing the larger placenta and lower weight ratio in ART and what downstream effects they have on development.

If the adverse outcome gene expression study were redesigned to be a full-scale study, it would need to include more samples and some exclusion criteria would need to be applied. It would be best to either narrow to a specific adverse outcome, such as small for gestational age term births, or to separate the different adverse outcomes into multiple, independent tests. As with the analyses in Chapter 3, a redesigned adverse outcome study would not directly compare twins to singletons and would ideally examine many more genetic regions using a genome-wide approach.

The database analysis, having fewer limitations and flaws in its design, is more in need of follow up than redesign. Because the results seem to suggest that the ART techniques themselves, particularly *in vitro* embryo culture, are affecting birth outcomes, it would be beneficial to more rigorously study the individual steps of the procedures. Culture medium composition, for instance, varies from one clinic to another, as there are numerous manufacturers of culture media, each using their own patent-protected formula. Comparing success rates and birth outcomes from different IVF clinics around the globe, considering the brand of culture medium they use as a factor, could offer more insight into how the process of *in vitro* culture is affecting gestation and which culture medium least affects birth outcomes. It is also important for more studies to be done that look at fertile couples who use ART for reasons other than infertility and couples who struggle with subfertility but still manage to conceive naturally. These couples offer a glimpse at the effects of ART and infertility independent of one another, something that is rare but very valuable in assessing the long-term safety of ART.

References

1. European Society of Human Reproduction and Embryology More than 8 million babies born from IVF since the world's first in 1978: European IVF pregnancy rates now steady at around 36 percent, according to ESHRE monitoring. ScienceDaily. Available via . https://www.sciencedaily.com/releases/2018/07/180703084127.htm. Accessed Jul 21, 2019

2. Finnström O, Källén B, Lindam A et al (2011) Maternal and child outcome after in vitro fertilization - a review of 25 years of population-based data from Sweden. Acta obstetricia et gynecologica Scandinavica 90(5):494-500. 10.1111/j.1600-0412.2011.01088.x

3. Henningsen AA, Pinborg A, Lidegaard Ø et al (2011) Perinatal outcome of singleton siblings born after assisted reproductive technology and spontaneous conception: Danish national sibling-cohort study. Fertility and Sterility 95(3):959-963. 10.1016/j.fertnstert.2010.07.1075

4. Davies MJ, Moore VM, Willson KJ et al (2012) Reproductive Technologies and the Risk of Birth Defects. New England Journal of Medicine 366(19):1803-1813. 10.1056/NEJMoa1008095

5. Palomba S, Homburg R, Santagni S et al (2016) Risk of adverse pregnancy and perinatal outcomes after high technology infertility treatment: a comprehensive systematic review. Reproductive Biology and Endocrinology 14(1):1-25

6. Barker DJP (1991) The foetal and infant origins of inequalities in health in Britain. J Public Health (Oxf) 13(2):64-68. 10.1093/oxfordjournals.pubmed.a042604

7. Hales CN, Barker DJ, Clark PM et al (1991) Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303(6809):1019-1022

8. Barker DJ, Bull AR, Osmond C et al (1990) Fetal and placental size and risk of hypertension in adult life. BMJ 301(6746):259-262

9. Eriksson JG, Forsen TJ, Kajantie E et al (2007) Childhood Growth and Hypertension in Later Life. Hypertension 49(6):1415-1421. 10.1161/HYPERTENSIONAHA.106.085597

10. Dunson DB, Baird DD, Colombo B (2004) Increased Infertility With Age in Men and Women. Obstetrics & Gynecology 103(1):51-56. 10.1097/01.AOG.0000100153.24061.45

11. Shah K, Sivapalan G, Gibbons N et al (2003) The genetic basis of infertility. Reproduction 126(1):13-25

12. Greenlee AR, Arbuckle TE, Chyou P (2003) Risk Factors for Female Infertility in an Agricultural Region. Epidemiology 14(4):429-436. 10.1097/01.EDE.0000071407.15670.aa

13. Benoff S, Jacob A, Hurley IR (2000) Male infertility and environmental exposure to lead and cadmium. Hum Reprod Update 6(2):107-121. 10.1093/humupd/6.2.107

14. The Practice Committee of the American Society for Reproductive Medicine (2004) Endometriosis and infertility. Fertility and Sterility 82:40-45. 10.1016/j.fertnstert.2004.06.016

15. Lissens W, Mercier B, Tournaye H et al (1996) Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. Hum Reprod 11(S4):55-80. 10.1093/humrep/11.suppl_4.55

16. Linsenmeyer TA (2000) Sexual function and infertility following spinal cord injury. Phys Med Rehabil Clin N Am 11(1):141-156

17. Rowe PJ (1999) Clinical aspects of infertility and the role of health care services. Reproductive Health Matters 7(13):103-111. 10.1016/S0968-8080(99)90119-2

18. Carlsen E, Giwercman A, Keiding N et al (1993) Evidence for decreasing quality of semen during past 50 years. International Journal of Gynecology & Obstetrics 41(1):112-113. 10.1016/0020-7292(93)90181-U

19. Levine H, Jørgensen N, Martino-Andrade A et al (2017) Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 23(6):646-659. 10.1093/humupd/dmx022

20. Tijani HA, Bhattacharya S (2010) The role of intrauterine insemination in male infertility. Human Fertility 13(4):226-232. 10.3109/14647273.2010.533811

21. Humaidan P, Alsbjerg B (2014) GnRHa trigger for final oocyte maturation: is HCG trigger history?. Reproductive BioMedicine Online 29(3):274-280. 10.1016/j.rbmo.2014.05.008

22. Glujovsky D, Farquhar C, Quinteiro Retamar AM et al (2016) Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. Cochrane Database Syst Rev(6):CD002118. 10.1002/14651858.CD002118.pub5

23. Maheshwari A, Hamilton M, Bhattacharya S (2016) Should we be promoting embryo transfer at blastocyst stage?. Reproductive BioMedicine Online 32(2):142-146

24. Cutting R (2018) Single embryo transfer for all. Best Practice & Research Clinical Obstetrics & Gynaecology 53:30-37. 10.1016/j.bpobgyn.2018.07.001

25. Wu Y, Chen W, Zhou L et al (2019) Single embryo transfer improve the perinatal outcome in singleton pregnancy. The Journal of Maternal-Fetal & Neonatal Medicine:1-6. 10.1080/14767058.2019.1571029

26. Fauser BC (2019) Towards the global coverage of a unified registry of IVF outcomes. Reproductive BioMedicine Online 38(2):133-137. 10.1016/j.rbmo.2018.12.001

27. Cavoretto P, Candiani M, Giorgione V et al (2018) Risk of spontaneous preterm birth in singleton pregnancies conceived after IVF/ICSI treatment: meta-analysis of cohort studies. Ultrasound in Obstetrics & Gynecology 51(1):43-53. 10.1002/uog.18930

28. McDonald SD, Han Z, Mulla S et al (2009) Preterm birth and low birth weight among in vitro fertilization singletons: A systematic review and meta-analyses. European Journal of Obstetrics & Gynecology and Reproductive Biology 146(2):138-148. 10.1016/j.ejogrb.2009.05.035

29. McDonald SD, Han Z, Mulla S et al (2010) Preterm birth and low birth weight among in vitro fertilization twins: A systematic review and meta-analyses. European Journal of Obstetrics & Gynecology and Reproductive Biology 148(2):105-113. 10.1016/j.ejogrb.2009.09.019

30. Haavaldsen C, Tanbo T, Eskild A (2012) Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536 567 pregnancies. Hum Reprod 27(2):576-582. 10.1093/humrep/der428

31. Daniel Y, Schreiber L, Geva E et al (1999) Do placentae of term singleton pregnancies obtained by assisted reproductive technologies differ from those of spontaneously conceived pregnancies?. Hum Reprod 14(4):1107-1110. 10.1093/humrep/14.4.1107

32. Yanaihara A, Hatakeyama S, Ohgi S et al (2018) Difference in the size of the placenta and umbilical cord between women with natural pregnancy and those with IVF pregnancy. J Assist Reprod Genet 35(3):431-434. 10.1007/s10815-017-1084-2

33. Vrooman L, Chao O, Bartolomei M (2017) Individual Assisted Reproduction Technology procedures induce abnormal placental development and reduced fetal weight in the mouse. Placenta 57:288. 10.1016/j.placenta.2017.07.207

34. de Waal E, Vrooman LA, Fischer E et al (2015) The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. Hum Mol Genet 24(24):6975-6985. 10.1093/hmg/ddv400

35. Cortessis VK, Azadian M, Buxbaum J et al (2018) Comprehensive meta-analysis reveals association between multiple imprinting disorders and conception by assisted reproductive technology. J Assist Reprod Genet 35(6):943-952. 10.1007/s10815-018-1173-x

36. Chiba H, Hiura H, Okae H et al (2013) DNA methylation errors in imprinting disorders and assisted reproductive technology. Pediatrics International 55(5):542-549. 10.1111/ped.12185

37. Odom LN, Segars J (2010) Imprinting disorders and assisted reproductive technology. Current Opinion in Endocrinology, Diabetes, and Obesity 17(6):517-522. 10.1097/MED.0b013e32834040a3

38. Uyar A, Seli E (2014) The impact of assisted reproductive technologies on genomic imprinting and imprinting disorders. Current Opinion in Obstetrics and Gynecology 26(3):210-221. 10.1097/GCO.0000000000000071

39. Rhon-Calderon EA, Vrooman LA, Riesche L et al (2019) The effects of Assisted Reproductive Technologies on genomic imprinting in the placenta. Placenta. 10.1016/j.placenta.2019.02.013

40. Jabbari K, Bernardi G (2004) Cytosine methylation and CpG, TpG (CpA) and TpA frequencies. Gene 333:143-149. 10.1016/j.gene.2004.02.043

41. Gardiner-Garden M, Frommer M (1987) CpG Islands in vertebrate genomes. Journal of Molecular Biology 196(2):261-282. 10.1016/0022-2836(87)90689-9

42. Aïssani B, Bernardi G (1991) CpG islands: features and distribution in the genomes of vertebrates. Gene 106(2):173-183. 10.1016/0378-1119(91)90197-J

43. Li E, Zhang Y (2014) DNA Methylation in Mammals. Cold Spring Harbor perspectives in biology 6(5):a019133. 10.1101/cshperspect.a019133

44. Henckel A, Nakabayashi K, Sanz LA et al (2009) Histone methylation is mechanistically linked to DNA methylation at imprinting control regions in mammals. Hum Mol Genet 18(18):3375-3383. 10.1093/hmg/ddp277

45. Delaval K, Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. Current Opinion in Genetics & Development 14(2):188-195. 10.1016/j.gde.2004.01.005

46. De Crescenzo A, Sparago A, Cerrato F et al (2013) Paternal deletion of the 11p15.5 centromeric-imprinting control region is associated with alteration of imprinted gene expression and recurrent severe intrauterine growth restriction. J Med Genet 50(2):99-103. 10.1136/jmedgenet-2012-101352

47. Du M, Zhou W, Beatty LG et al (2004) The KCNQ1OT1 promoter, a key regulator of genomic imprinting in human chromosome 11p15.5. Genomics 84(2):288-300. 10.1016/j.ygeno.2004.03.008

48. Verona RI, Mann MRW, Bartolomei MS (2003) Genomic imprinting: intricacies of epigenetic regulation in clusters. Annual Review Of Cell And Developmental Biology 19:237-259

49. Wilkins JF, Haig D (2003) What good is genomic imprinting: the function of parent-specific gene expression. Nature Reviews Genetics 4(5):359-368. 10.1038/nrg1062

50. Tycko B, Morison IM (2002) Physiological functions of imprinted genes. Journal of Cellular Physiology 192(3):245-258. 10.1002/jcp.10129

51. Monk D (2015) Genomic imprinting in the human placenta. American Journal of Obstetrics and Gynecology 213(4):S162. 10.1016/j.ajog.2015.06.032

52. Sanchez-Delgado M, Court F, Vidal E et al (2016) Human Oocyte-Derived Methylation Differences Persist in the Placenta Revealing Widespread Transient Imprinting. PLoS Genetics 12(11)

53. Monteagudo-Sánchez A, Sánchez-Delgado M, Mora JRH et al (2019) Differences in expression rather than methylation at placenta-specific imprinted loci is associated with intrauterine growth restriction. Clin Epigenetics 11(1):35. 10.1186/s13148-019-0630-4

54. Eggermann T, Nanclares GPd, Maher ER et al (2015) Imprinting disorders: a group of congenital disorders with overlapping patterns of molecular changes affecting imprinted loci. Clinical Epigenetics. Available via . http://link.galegroup.com.ezproxy.library.ubc.ca/apps/doc/A445250136/HRCA?sid=googlescholar. Accessed Jul 21, 2019

55. Butler MG (2009) Genomic imprinting disorders in humans: a mini-review. J Assist Reprod Genet 26(9-10):477-486. 10.1007/s10815-009-9353-3

56. Li H, Zhao P, Xu Q et al (2016) The autism-related gene SNRPN regulates cortical and spine development via controlling nuclear receptor Nr4a1. Scientific reports 6(1):29878. 10.1038/srep29878

57. Docherty LE, Kabwama S, Lehmann A et al (2013) Clinical presentation of 6q24 transient neonatal diabetes mellitus (6q24 TNDM) and genotype-phenotype correlation in an international cohort of patients. Diabetologia 56(4):758-762. 10.1007/s00125-013-2832-1

58. Stelzer G, Rosen N, Plaschkes I et al (2016) The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. Current Protocols in Bioinformatics 54(1):1.30.33. 10.1002/cpbi.5

59. Elhamamsy AR (2017) Role of DNA methylation in imprinting disorders: an updated review. J Assist Reprod Genet 34(5):549-562. 10.1007/s10815-017-0895-5

60. Bowdin S, Allen C, Kirby G et al (2007) A survey of assisted reproductive technology births and imprinting disorders. Hum Reprod 22(12):3237-3240. 10.1093/humrep/dem268

61. Popp C, Dean W, Feng S et al (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463(7284):1101-1105. 10.1038/nature08829

62. Lucifero D, Mann MRW, Bartolomei MS et al (2004) Gene-specific timing and epigenetic memory in oocyte imprinting. Hum Mol Genet 13(8):839-849. 10.1093/hmg/ddh104

63. Hiura H, Okae H, Chiba H et al (2014) Imprinting methylation errors in ART. Reprod Med Biol 13(4):193-202. 10.1007/s12522-014-0183-3

64. Sato A, Otsu E, Negishi H et al (2007) Aberrant DNA methylation of imprinted loci in superovulated oocytes. Hum Reprod 22(1):26-35. 10.1093/humrep/del316

65. Fauque P (2013) Ovulation induction and epigenetic anomalies. Fertility and Sterility 99(3):616-623. 10.1016/j.fertnstert.2012.12.047

66. Fortier AL, Lopes FL, Darricarrère N et al (2008) Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. Hum Mol Genet 17(11):1653-1665. 10.1093/hmg/ddn055

67. Smith ZD, Chan MM, Humm KC et al (2014) DNA methylation dynamics of the human preimplantation embryo. Nature 511(7511):611-615. 10.1038/nature13581

68. Guo H, Zhu P, Yan L et al (2014) The DNA methylation landscape of human early embryos. Nature 511(7511):606-610. 10.1038/nature13544

69. el Hajj N, Haaf T (2013) Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. Fertility and Sterility 99(3):632-641. 10.1016/j.fertnstert.2012.12.044

70. Grafodatskaya D, Cytrynbaum C, Weksberg R (2013) The health risks of ART. Science & Society 14(2):129-135

71. Canovas S, Ross PJ, Kelsey G et al (2017) DNA Methylation in Embryo Development: Epigenetic Impact of ART (Assisted Reproductive Technologies). BioEssays 39(11):1700106. 10.1002/bies.201700106

72. Lazaraviciute G, Kauser M, Bhattacharya S et al (2014) A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI compared with children conceived spontaneously. Hum Reprod Update 20(6):840-852. 10.1093/humupd/dmu033

73. Choux C, Binquet C, Carmignac V et al (2018) The epigenetic control of transposable elements and imprinted genes in newborns is affected by the mode of conception: ART versus

spontaneous conception without underlying infertility. Hum Reprod 33(2):331-340. 10.1093/humrep/dex366

74. Gentilini D, Somigliana E, Pagliardini L et al (2018) Multifactorial analysis of the stochastic epigenetic variability in cord blood confirmed an impact of common behavioral and environmental factors but not of in vitro conception. Clinical Epigenetics. Available via . http://link.galegroup.com.ezproxy.library.ubc.ca/apps/doc/A546893081/HRCA?sid=googlescholar. Accessed Jul 21, 2019

75. Ghosh J, Coutifaris C, Sapienza C et al (2017) Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. Clinical Epigenetics 9(1). 10.1186/s13148-017-0318-6

76. Katagiri Y, Aoki C, Tamaki-Ishihara Y et al (2010) Effects of Assisted Reproduction Technology on Placental Imprinted Gene Expression. Obstetrics and Gynecology International 2010:e437528. 10.1155/2010/437528

77. Katari S, Turan N, Bibikova M et al (2009) DNA methylation and gene expression differences in children conceived in vitro or in vivo. Hum Mol Genet 18(20):3769-3778. 10.1093/hmg/ddp319

78. Li L, Wang L, Le F et al (2011) Evaluation of DNA methylation status at differentially methylated regions in IVF-conceived newborn twins. Fertility and Sterility 95(6):1975-1979. 10.1016/j.fertnstert.2011.01.173

79. Litzky JF, Deyssenroth MA, Everson TM et al (2017) Placental imprinting variation associated with assisted reproductive technologies and subfertility. Epigenetics 12(8):653-661. 10.1080/15592294.2017.1336589

80. Nelissen ECM, Dumoulin JCM, Daunay A et al (2013) Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the H19 and MEST differentially methylated regions. Human Reproduction 28(4):1117-1126. 10.1093/humrep/des459

81. Nelissen ECM, Dumoulin JCM, Busato F et al (2014) Altered gene expression in human placentas after IVF/ICSI. Hum Reprod 29(12):2821-2831. 10.1093/humrep/deu241

82. Rancourt RC, Harris HR, Michels KB (2012) Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. Hum Reprod 27(7):2208-2216. 10.1093/humrep/des151

83. Song S, Ghosh J, Mainigi M et al (2015) DNA methylation differences between in vitro- and in vivo-conceived children are associated with ART procedures rather than infertility. Clinical Epigenetics 7(1). 10.1186/s13148-015-0071-7

84. Zheng H, Shi X, Wang L et al (2011) Study of DNA methylation patterns of imprinted genes in children born after assisted reproductive technologies reveals no imprinting errors: A pilot study. Exp Ther Med 2(4):751-755. 10.3892/etm.2011.261

85. Apgar V (1966) The Newborn (APGAR) Scoring System: Reflections and Advice. Pediatric Clinics of North America 13(3):645-650. 10.1016/S0031-3955(16)31874-0

86. Salafia CM, Charles AK, Maas EM (2006) Placenta and Fetal Growth Restriction : Clinical Obstetrics and Gynecology. Clinical Obstetrics and Gynecology 49(2):236-256

87. Eskild A, Monkerud L, Tanbo T (2013) Birthweight and placental weight; do changes in culture media used for IVF matter? Comparisons with spontaneous pregnancies in the corresponding time periods. Hum Reprod 28(12):3207-3214. 10.1093/humrep/det376

88. Zhu J, Lin S, Li M et al (2014) Effect of in vitro culture period on birthweight of singleton newborns. Hum Reprod 29(3):448-454. 10.1093/humrep/det460

89. Nelissen EC, Van Montfoort AP, Coonen E et al (2012) Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. Hum Reprod 27(7):1966-1976. 10.1093/humrep/des145

90. Shih W, Rushford DD, Bourne H et al (2008) Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse effect of oocyte collection. Hum Reprod 23(7):1644-1653. 10.1093/humrep/den150

91. Pinborg A, Loft A, Henningsen AA et al (2010) Infant outcome of 957 singletons born after frozen embryo replacement: The Danish National Cohort Study 1995–2006. Fertility and Sterility 94(4):1320-1327. 10.1016/j.fertnstert.2009.05.091

92. Wennerholm U, Henningsen AA, Romundstad LB et al (2013) Perinatal outcomes of children born after frozen-thawed embryo transfer: a Nordic cohort study from the CoNARTaS group. Hum Reprod 28(9):2545-2553. 10.1093/humrep/det272

93. Maheshwari A, Pandey S, Shetty A et al (2012) Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. Fertility and Sterility 98(2):377.e9. 10.1016/j.fertnstert.2012.05.019

94. Pelkonen S, Koivunen R, Gissler M et al (2010) Perinatal outcome of children born after frozen and fresh embryo transfer: the Finnish cohort study 1995–2006. Hum Reprod 25(4):914-923. 10.1093/humrep/dep477

95. Kramer MS, Platt RW, Wen SW et al (2001) A new and improved population-based Canadian reference for birth weight for gestational age. Pediatrics 108(2):E35. 10.1542/peds.108.2.e35

96. Alexander GR, Himes JH, Kaufman RB et al (1996) A united states national reference for fetal growth. Obstetrics & Gynecology 87(2):163-168. 10.1016/0029-7844(95)00386-X

97. Niklasson A, Ericson A, Fryer JG et al (1991) An Update of the Swedish Reference Standards for Weight, Length and Head Circumference at Birth for Given Gestational Age (1977-1981). Acta Paediatrica 80(8-9):756-762. 10.1111/j.1651-2227.1991.tb11945.x

98. Fenton TR, Kim JH (2013) A systematic review and meta-analysis to revise the Fenton growth chart for preterm infants. BMC Pediatr 13(1):1-13. 10.1186/1471-2431-13-59

99. Hoffman CS, Messer LC, Mendola P et al (2008) Comparison of gestational age at birth based on last menstrual period and ultrasound during the first trimester. Paediatric and Perinatal Epidemiology 22(6):587-596. 10.1111/j.1365-3016.2008.00965.x

100. Kieler H, Axelsson O, Nilsson S et al (1995) The length of human pregnancy as calculated by ultrasonographic measurement of the fetal biparietal diameter. Ultrasound in Obstetrics & Gynecology 6(5):353-357. 10.1046/j.1469-0705.1995.06050353.x

101. Teixeira C (2014) Living on the "edge of the suburbs" of Vancouver: A case study of the housing experiences and coping strategies of recent immigrants in Surrey and Richmond. The Canadian Geographer 58(2):168-187. 10.1111/j.1541-0064.2013.12055.x

102. Parker JD, Schoendorf KC, Kiely JL (1994) Associations between measures of socioeconomic status and low birth weight, small for gestational age, and premature delivery in the United States. Annals of Epidemiology 4(4):271-278. 10.1016/1047-2797(94)90082-5

103. Luque-Fernandez MA, Ananth CV, Jaddoe VWV et al (2015) Is the fetoplacental ratio a differential marker of fetal growth restriction in small for gestational age infants?. European Journal of Epidemiology 30(4):331-341. 10.1007/s10654-015-9993-9

104. Mouillet J, Chu T, Sadovsky Y (2011) Expression patterns of placental microRNAs. Birth Defects Research Part A: Clinical and Molecular Teratology 91(8):737-743. 10.1002/bdra.20782

105. Bloise E, Lin W, Liu X et al (2012) Impaired Placental Nutrient Transport in Mice Generated by in Vitro Fertilization. Endocrinology 153(7):3457-3467. 10.1210/en.2011-1921

106. Vincent RN (2015) Investigation into DNA methylation and gene expression profiles of imprinted regions in infants conceived via assisted reproductive technologies, University of British Columbia

107. Varrault A, Gueydan C, Delalbre A et al (2006) Zac1 Regulates an Imprinted Gene Network Critically Involved in the Control of Embryonic Growth. Developmental Cell 11(5):711-722. 10.1016/j.devcel.2006.09.003

108. Yang AS, Estécio MRH, Doshi K et al (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38. 10.1093/nar/gnh032

109. Weisenberger DJ, Campan M, Long TI et al (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823-6836. 10.1093/nar/gki987

110. Nancy J. Smilinich, Colleen D. Day, Galina V. Fitzpatrick et al (1999) A Maternally Methylated CpG Island in KvLQT1 Is Associated with an Antisense Paternal Transcript and Loss of Imprinting in Beckwith-Wiedemann Syndrome. Proceedings of the National Academy of Sciences of the United States of America 96(14):8064-8069. 10.1073/pnas.96.14.8064

111. Lao TT, Wong WM (1996) Placental ratio and intrauterine growth retardation. BJOG: An International Journal of Obstetrics & Gynaecology 103(9):924-926. 10.1111/j.1471-0528.1996.tb09914.x

112. Huntriss JD, Hemmings KE, Hinkins M et al (2013) Variable imprinting of the MEST gene in human preimplantation embryos. European Journal Of Human Genetics: EJHG 21(1):40-47. 10.1038/ejhg.2012.102

113. Hiura H, Hattori H, Kobayashi N et al (2017) Genome-wide microRNA expression profiling in placentae from frozen-thawed blastocyst transfer. Clinical epigenetics 9(1):79. 10.1186/s13148-017-0379-6

114. Ji M, Wang X, Wu W et al (2018) ART manipulation after controlled ovarian stimulation may not increase the risk of abnormal expression and DNA methylation at some CpG sites of H19,IGF2 and SNRPN in foetuses: a pilot study. Reproductive biology and endocrinology : RB&E 16(1):63. 10.1186/s12958-018-0344-z

115. Yap C, Loh MT, Heng KK et al (2000) Variability in CD34+ Cell Counts in Umbilical Cord Blood:Implications for Cord Blood Transplants. GOI 50(4):258-259. 10.1159/000010327

116. Robertson KD (2005) DNA methylation and human disease. Nature Reviews Genetics 6(8)

117. Amor DJ, Halliday J (2008) A review of known imprinting syndromes and their association with assisted reproduction technologies. Hum Reprod 23(12):2826-2834. 10.1093/humrep/den310

118. Steuerwald NM, Bermúdez MG, Wells D et al (2007) Maternal age-related differential global expression profiles observed in human oocytes. Reproductive BioMedicine Online 14(6):700-708. 10.1016/S1472-6483(10)60671-2

119. Munné S, Sandalinas M, Escudero T et al (2002) Chromosome mosaicism in cleavage-stage human embryos: evidence of a maternal age effect. Reproductive BioMedicine Online 4(3):223-232. 10.1016/S1472-6483(10)61810-X

120. Yousefi P, Huen K, Davn V et al (2015) Sex differences in DNA methylation assessed by 450 K BeadChip in newborns. BMC Genomics 16(908)

121. Ellegren H, Parsch J (2007) The evolution of sex-biased genes and sex-biased gene expression. Nature Reviews Genetics 8(9):689-698. 10.1038/nrg2167

122. Xu N, Barlow GM, Cui J et al (2017) Comparison of Genome-Wide and Gene-Specific DNA Methylation Profiling in First-Trimester Chorionic Villi From Pregnancies Conceived With Infertility Treatments. Reprod Sci 24(7):996-1004. 10.1177/1933719116675056