Infertility and infertility treatment: childhood cancer and epigenetic risks

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Dedication

This dissertation is dedicated to the memory of my father who instilled in me the belief that I could do anything I chose to do.

Abstract

Although childhood cancer is rare, it can have a devastating impact on the children who develop it and their families. Just as in adults, cancer in children is comprised of many disease types. Since the window for exposure is quite limited in childhood cancer, exposures encountered *in utero* are likely to contribute to carcinogenesis. Thus, etiologic studies have focused on exposures that occur during pregnancy for cancers that arise early in life.

Parental infertility and infertility treatment have been hypothesized as possible risk factors for childhood cancer. The link is suspected since many infertile couples may have specific genetic or epigenetic anomalies that could be passed on to their children, which in turn could lead to carcinogenesis. Infertility treatment occurs at or near conception and may persist for several days after conception, making it plausible for treatment to affect early embryo development. In addition, infertility or infertility treatment may alter epigenetic patterns in the developing embryo resulting in an increase risk of childhood cancer. All of these reasons suggest a need to examine infertility and its treatment further.

Three related studies are combined in this thesis to examine the potential influence of infertility and infertility treatment on childhood cancer. The first two studies examined the potential link between infertility and its treatment and two specific childhood cancer diagnoses: infant leukemia and germ cell tumors (GCT). As these two diagnoses are quite rare, few previous studies have been performed to examine infertility or its treatment as a possible risk factor. Overall, no significant associations between

infertility or its treatment and infant leukemia or childhood GCT were found. However, some notable subgroup associations were found in both studies. In infant leukemia, there was an increased risk of the rare MLL- subtype in children born to women not trying to conceive compared to those trying to conceive for less than one year for all types combined and for acute lymphoblastic leukemia (ALL). In contrast, there was a decreased risk of acute myeloid leukemia (AML) for children born to women who reported use of medication to help them become pregnant. In GCT, there was an increased risk for non-gonadal tumors in females born to women with at least two fetal losses.

The final study examined DNA methylation as a potential mechanism by which assisted reproductive technology (ART) might influence the risk of childhood cancer. The data were consistent with no difference in methylation between groups at all loci for lymphocyte samples. Possible differences were found in buccal cell samples for two loci; IGF2 DMR0 and IGF2R. Subgroup analysis indicated potential lower methylation in those whose parents used ART for unexplained infertility. Correlation between lymphocyte and buccal cell samples was low for all loci.

The combined results of all three studies indicate no increased risk of infant leukemia, pediatric germ cell tumors, or epigenetic disruptions in specific loci associated with Beckwith-Wiedemann syndrome and certain types of childhood cancer. While an association may still exist for different types of childhood cancer or methylation levels in other loci or tissues, these studies should reassure parents of children conceived through infertility treatment at least somewhat.

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ART	Assisted reproductive technology
AS	Angelman syndrome
BC	Birth certificate
BWS	Beckwith-Wiedemann syndrome
CCG	Children's Cancer Group
CI	Confidence interval
COG	Children's Oncology Group
DCRU	Delaware clinical research unit
DMR	Differentially methylated region
DNMT	DNA methyltransferase
GCT	Germ cell tumor
GEE	Generalized estimating equations
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IMPART	Imprinting and methylation patterns after ART
IUI	Intrauterine insemination
IVF	In vitro fertilization
LCA	Latent class analysis
LOI	Loss of imprinting

MLL	Mixed lineage leukemia
ncRNA	Non-coding RNA
OR	Odds ratio
OS	Oxidative stress
RDD	Random digit dialing
ROS	Reactive oxygen species
SC	Spontaneous conception
SRS	Silver-Russell syndrome
UMRMC	University of Minnesota Reproductive Medicine Center
UPD	Uniparental disomy
US	United States

Introduction

The use of infertility treatment is rapidly rising. For example, assisted reproductive technology (ART), which primarily consists of in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI), now accounts for over 50,000 children born each year in the United States (US)[1]. Other infertility treatments contribute substantially as well, including ovulation stimulating drugs. The percent of children born to mothers using ovulation stimulating drugs in the absence of ART was estimated to be 4.6% of all children born in the US in 2005[2]. With such a large number of children conceived through ART, ovulation stimulation, and other treatments, research into possible adverse effects is of the utmost importance.

A particular concern is childhood cancer, which is a devastating disease both for the child and the child's family, and has been hypothesized to be associated with parental infertility or its treatment. There are several possible pathways between infertility or its treatment and childhood cancer. Infertility itself may confer upon the parents genetic or epigenetic anomalies that can be transmitted to future generations. Infertility treatment may also induce genetic or epigenetic anomalies in early embryonic and fetal development. Pathways involving epigenetic mechanisms may be the most plausible link between ART and childhood cancer.

The potential for epigenetic disruptions in children born after infertility treatment was first seen in a number of studies showing an increase in imprinting disorders in these children[3-12]. These studies further indicated that the cause of the imprinting disorders was due to epigenetic disruptions rather than mutations or uniparental disomy (UPD) [5, 6, 8, 10-

[17]. In addition to their role in imprinting disorders, epigenetic factors, in particular DNA methylation and genomic imprinting, control gene expression and cell proliferation, and are frequently seen in cancerous tissue samples. Tumor cells have overall hypomethylation, which occurs in repetitive elements, causing instability and predisposes DNA to further mutations and damage[18]. Another change in methylation leading to carcinogenesis is hypermethylation of particular genes that effectively silences gene transcription. This silencing occurs in DNA repair and cell-cycle regulation genes, causing abnormal growth and unchecked DNA damage[18].

Some gene regions are associated with both cancer and imprinting disorders. These regions are of particular interest in the study of children born after infertility treatment given the mounting evidence of an association between these treatments and some imprinting disorders. For example, one set of genes, IGF2 and H19, is associated with the imprinting disorder Beckwith-Wiedemann syndrome (BWS), childhood cancers (e.g. Wilms tumor, hepatoblastoma, and pediatric testicular germ cell tumors), and adult cancers (e.g. colorectal, bladder, and breast cancer)[19-21]. Epigenetic changes on a global scale or within particular genes could be an early sign of carcinogenesis and have been proposed as early diagnostic markers of disease[22, 23].

Several studies examining the association between infertility or infertility treatment and childhood cancer have been conducted. Both cohort studies and case-control studies have been performed with differing results. Most cohort studies found that observed cases of childhood cancer in children conceived through ART were similar to the expected number of cases[24-29]. However, these studies were based on relatively

few observed cases and short follow-up times. In case-control studies, a potential increase in risk has been found for leukemia, hepatoblastoma, neuroblastoma, and retinoblastoma for children conceived through use of infertility treatment or after a period of parental infertility[30-32]. These case-control studies, however, were prone to recall and selection bias, and have used imprecise measures of parental infertility or its treatment. Additional research is needed to further explore potential risks in specific cancer types.

The three studies that make up this thesis combine evidence from traditional and molecular epidemiology to explore the association between childhood cancer, epigenetic disruptions, and parental infertility. The first two studies focus on specific rare types of childhood cancer to examine the association between markers of parental infertility and each cancer type. The third study examines the potential for epigenetic disruptions in genes associated with imprinting disorders and cancer in children born after ART. Taken as a whole these studies will add to the growing literature that examines potential adverse events in children born after parental infertility or its treatment.

Chapter 1

Background: Infant leukemia, pediatric germ cell tumors, and infertility

Childhood cancer and parental infertility

Infertility is generally defined as failure to conceive after one year or more of trying. Both parental infertility and its treatment have been thought to be potential risk factors for childhood cancer. First, infertility may have adverse consequences for subsequent offspring due to the increased potential of parents to have genetic or epigenetic anomalies that could be passed on to their offspring. In addition, treatments for infertility that occur around the time of fertilization, such as ovulation stimulation, may foster alteration in epigenetic patterns in the developing embryo. These changes in genetic or epigenetic factors could lead to cancer development in children born after infertility or infertility treatment.

Many studies have been conducted to examine the association between infertility or infertility treatment and childhood cancer. Several large cohort studies have examined ART and childhood cancer, but few have looked at specific cancer diagnoses. Most studies have found that the total number of cancer cases observed in a cohort of children born after ART were similar to what was expected[24-29], but were based on small numbers of cancers overall and relatively short follow-up times. Case-control studies have examined infertility in general and have found some increase in risk for leukemia, hepatoblastoma, neuroblastoma, and retinoblastoma[30-32]. Rarer childhood cancer diagnoses or subsets of diagnoses have not been frequently examined with respect to infertility or infertility treatment.

Infant Leukemia

With approximately 40 cases per million infants diagnosed in the U.S. in 1992-2004 infant leukemia is a rare form of childhood cancer[33]. Infant leukemia is a distinct subtype of childhood leukemia with differences in type of diagnosis, clinical symptoms, tumor genetics, and response to treatment [34]. Since the latency period is extremely short in infant leukemia the relevant exposure(s) most likely occur *in utero*. Thus the periconceptional period is an important time frame in searching for etiologic factors.

There are several features of infant leukemia that make it unique and might have an impact on its etiology. First, the distribution of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) subtypes is very different in infant leukemia compared to childhood leukemia overall. While there are more than four times as many ALL diagnoses compared to AML diagnoses in children under 18 years, the ratio of ALL to AML in infants is about 1.3 in the U.S[33]. Another feature that sets infant leukemia apart is that the majority of cases have somatic mutations in the mixed lineage leukemia (MLL) gene. In infant leukemia, about 80% of ALL and 50% of AML have a recombination event within the MLL gene present in the leukemia cells[35]. In addition, mutations in the MLL gene appear to occur *in utero* since rearrangements in the MLL gene have been identified in neonatal blood spots collected at birth[36, 37]. The distinction between subtype and MLL status is important in infant leukemia since many studies indicate a potential difference in risk factors for disease based on these subgroups, thus most studies examine each combination of subtype and MLL status separately.

One specific class of exposures has been gaining interest as a potential mechanism for MLL translocations: DNA topoisomerase II inhibitors. These were suggested to be leukemiogenic due to an increase in AML after treatment with certain chemotherapeutic drugs. A specific form of leukemia with MLL gene rearrangements was found to occur after treatment with epipodophyllotoxins, which target DNA topoisomerase II[38]. DNA, when not replicating, is tightly coiled for packaging into each cell[39]. For replication and transcription, however, it is necessary for the DNA to be "opened up" and the two strands separated. The role of DNA topoisomerases is to facilitate this separation by cutting the DNA strands. Once the DNA has been transcribed or replicated, the break caused by DNA topoisomerase is rejoined. DNA topoisomerase II inhibitors bind with the topoisomerase II enzyme and inhibit the rejoining of the DNA strands causing a permanent break in the DNA[39]. Generally, this inhibition results in cell death, but in some cases the result is an illegitimate recombination event. This event could result in leukemogenesis[39].

Little is known from epidemiological studies about the possible risk factors for infant leukemia. However, some studies have indicated a possible relationship with high birth weight[40, 41], prior fetal loss[42], maternal alcohol consumption during pregnancy[43], DNA damaging drugs[44], maternal exposure to mind altering drugs[45], maternal consumption of dietary DNA topoisomerase-II inhibitors[46, 47], and maternal exposure to household pesticides, dipyrone, and hormones[48]. Most findings have not been replicated, however, since infant leukemia is rare and few studies have been conducted.

Specifically, DNA topoisomerase II inhibitors have been the focus of a few studies of infant leukemia because of their link to MLL rearrangements. Potential inhibitors are not limited to chemotherapy drugs, but are also found in certain foods such as coffee, tea, wine, chocolate, some fruits and vegetables, and soy. Some chemicals are also DNA topoisomerase inhibitors including permethrin, benzene, and thiram[34]. In studies examining dietary sources of DNA topoisomerase II inhibitors, an increased risk for infant AML was observed in one[46], while a second study with MLL data found a potential increased risk limited to infants with AML and an MLL rearrangement[47]. Non-dietary DNA topoisomerase II inhibitors have not been extensively studied with regard to infant leukemia. Two studies that proposed to examine these types of exposure were problematic due to the rarity of these exposures and measurement problems[44, 48]. Studies in children and adults have indicated some increased risk of leukemia with increased exposure to benzene and pesticides (reviewed in [49]).

Additional factors could also lead to DNA damage *in utero* and possible MLL translocations or other cytogenetic defects. High birth weight has been long associated with childhood leukemia and with infant leukemia in several studies. The common hypothesis associated with this observation is an increased expression of insulin-like growth factor 1 leading to increased growth and an increased risk for mutations[50]. In fact, one study recently linked high birth weight to MLL translocations suggesting a possible common cause[51]. Alcohol consumption during pregnancy might also result in DNA damage. A recent study in mice indicated that larger doses in pregnancy resulted in DNA damage in the offspring [52]. Another study in rats indicated that ethanol given to

fetal rats caused the generation of reactive oxygen species and lipid peroxidation[53]. In one study, which found an increased risk of infant leukemia in women who used hormones during pregnancy (oral contraceptives, hormones for pregnancy retention, and thyroid hormones), a potential for DNA damage was discussed[48]. This study suggested that semiquinone and quinine, which are products of estrogen biosynthesis, may cause single strand DNA breaks.

Only one previous study in infants has examined the possible association between infant leukemia and parental infertility or infertility treatment. This study surveyed medication recorded in the mother's medical record during pregnancy and found a non-significant inverse association with clomiphene, an ovarian stimulant, but the analysis was only based on two exposed cases[54]. Studies in older children that examined indication or treatment of infertility and childhood leukemia have been mixed with some finding an indication of increased risk [31, 55-57] and others finding no association [45, 58-60]. However, all suffered from relatively low power since there were few cases or controls reporting infertility or infertility treatment. Table 1 provides the details of previous studies (both infant and childhood leukemia) and a potential association with infertility and/or infertility treatment.

Pediatric Germ Cell Tumors

Childhood germ cell tumors (GCT), while all originating in the primordial germ cell, are a collection of tumors with a variety of histological types[61]. Incidence of GCT overall is about 11.5 per million children but varies with age and has two notable peaks,

one during the first 12 months of life and one during adolescence and early adulthood[33]. GCTs occur both in the gonadal region and in other areas of the body including the sacrococcygeal, mediastinal, and intracranial regions.

Little is know about the etiology of this rare group of cancers. In studies of GCT performed in children under the age of 15 years, few potential risk factors have been consistent. One study out of the United Kingdom linked incidence of pediatric GCT with major malformations [62]. Another study on childhood testicular cancer found a marginal association with genitourinary defects [63]. Two studies have also been conducted in the United States; one, an exploratory analysis (Children's Cancer Group Study (CCGS)) and the other, the current study being investigated (Children's Oncology Group (COG) study). The analysis of the COG study to date indicates no notable relationship with residential pesticides, parental occupational exposure to pesticides, parental smoking and drinking, family history of cancer, maternal vitamin supplementation, or exposure to female hormones during pregnancy, but, among males, did find an association with undescended testis[64-70]. The CCGS found positive associations with increasing number of live births, low gestational age, high birth weight, maternal urinary infection during pregnancy, maternal or paternal exposure to chemicals or solvents, maternal exposure to plastic or resin fumes, and appendectomy in the index child[71]. Two other studies have included children in a wide age range of GCT. One of these examined testicular cancers and found a decreasing risk for increasing maternal parity for children diagnosed at ages 0-4[72]. The other study did not examine younger age groups separately[73].

Some factors potentially related to parental infertility have been frequently observed in older adolescents and adults with GCT, specifically testicular GCT. In particular, an increased risk has been observed for cryptorchidism[74-79] and chromosomal abnormalities (particularly sex chromosome abnormalities)[80, 81]. Maternal exposure to hormones during pregnancy has been linked to GCT in some studies[74, 75, 82, 83], while others have found no association[84, 85]. These associations are interesting, however, it is not clear that they apply to GCT diagnosed in children since the tumor types are different in young children compared to older adolescents and adults. In young children testicular GCT are mainly yolk sac tumors with loss of chromosome 6q, deletions of chromosome 1p, and abnormalities in chromosomes 2 and 3p, whereas post-pubertal testicular GCT are primarily seminomas and non-seminomas with an isochromosome of the short arm of chromosome 12[86]. These disparate tumor types and genetic abnormalities may suggest different etiologies. In addition, a large proportion of the tumors in younger children are not testicular GCT, but are rather ovarian or non-gonadal GCT[61]. Little is known about potential risk factors in non-gonadal GCT, which arise due to aberrant germ cell migration early in gestation[87]. The mechanisms through which this migration occurs have not been fully elucidated so it is difficult to speculate on possible causes for abnormal migration that could lead to non-gonadal GCT[88]. Thus, it is unclear that the adult testicular cancer literature can provide much insight in to childhood GCT.

Parental infertility and infertility treatment have not often been studied in pediatric GCT. History of infertility was examined briefly in the COG study and no relationship was found for all GCT cases combined (OR = 1.08, 95% CI: 0.54-2.18)[67].

However, the variable used to assess history of infertility was not described and the subgroup analysis was not performed. The COG study also examined exposure to maternal hormones during pregnancy and found no evidence for an association (OR = 1.15, 95% CI: 0.63-2.12). One other study examined exposure to maternal hormones during pregnancy. This study included women up to the age of 35 years and only examined ovarian GCT, but did find an increased risk for exposure to estrogenic hormones in the first trimester of pregnancy[73].

Biological Mechanisms linking infertility and childhood cancer

Causes of infertility

Infertility affects a large number of couples in the U.S. In 2002, it was estimated that 2.1 million married women were infertile (defined as no pregnancy after 12 months or more of not using contraception)[89]. In addition, about 12% of women of reproductive age indicated that they had ever used some kind of medical assistance to help to become pregnant or to maintain a pregnancy[89]. The use of and success of infertility treatment has grown tremendously due to advances in treatment and insurance coverage[90-92]. Between 1996 and 2005, the number of children born after ART has increased 150% and now accounts for over 1% of all children born in the U.S[93].

Infertility could be due to a number of different factors, which may originate either in the male or female partner. Maternal factors for infertility include advanced maternal age, tubal factors (post-infection tubal damage, tubal obstruction, pelvic adhesions), ovulatory dysfunctions (including polycystic ovary syndrome), and

endometriosis[94]. Male factor infertility includes varicocele, immunological problems (antisperm antibodies), ejaculatory dysfunction, or defective sperm parameters (low concentration, poor motility, or abnormal morphology)[95]. Although estimates vary widely, in about 10-17% of couples no cause is identified[96, 97]. There are also a large number of possible contributing factors that enhance or help to cause infertility. Some of the more well documented factors include untreated sexually transmitted disease such Chlamydia, hormonal imbalances, smoking, NSAID use, radiation exposure, exposure to certain chemicals including pesticides, exposure to lead or other heavy metals, caffeine consumption, and obesity[94, 95, 98-101]. *With the wide diversity of types of infertility and many contributing factors, one of two things (or both) must be true in order to clearly identify infertility in general as a risk factor for childhood cancer*; either the treatment is the important etiologic factor, or there is some underlying unifying etiologic factor for all (or at least a substantial proportion of) causes of infertility. For example, one potential unifying theory of an underlying commonality is oxidative stress (OS), which may be associated with many observed infertility factors, and also contributes directly to infertility through various mechanisms. Thus, both OS and infertility treatment could be the most direct, putative cause for cancer risk in children born to parents with infertility or who received treatment for infertility.

Oxidative stress

While reactive oxygen species (ROS) are a normal part of cellular functioning, high levels of ROS, past the levels that can be detoxified by antioxidants and other

mechanisms, can lead to OS and ultimately DNA damage[102]. OS and subsequent damage is implicated in several diseases including cancer, cardiovascular disease, and Alzheimer's disease[102]. Many studies have shown an increase in OS levels in both women and men with infertility. In women, OS has been linked in some studies with PCOS, endometriosis, hydrosalpinx, unexplained infertility, and recurrent pregnancy loss although the literature is not completely consistent[103, 104]. In sperm cells, ROS has been shown to cause DNA damage through base modification, deletions, frame shifts, and chromosomal rearrangements[105, 106]. Normally, damage to the sperm DNA would be repaired through internal mechanisms in the sperm or by the oocyte after fertilization. However, due to problems in DNA repair capacity in the sperm and oocyte the damage may remain and be passed on to the zygote[107].

In addition to contributing to infertility, OS could be further exacerbated through treatment for infertility. In ART, exposure to ROS is increased through sperm preparation techniques that use centrifugation to separate sperm from seminal plasma[108]. Another potential source of ROS in infertility treatment is the culture media, although little study has been done to examine how this may impact DNA integrity in the pre-implantation embryo[109]. Finally, sperm selected through intracytoplasmic sperm injection (ICSI) could have damage caused by ROS since the normal selection process for sperm is bypassed. Thus, there may be a greater chance of fertilization with sperm that exhibits DNA damage caused by ROS, even though much care is taken to select sperm with normal morphology as these inspection methods may be crude compared to the level of DNA damage associated with ROS.

There is also a link between increases in ROS and epigenetic modifications. ROS can have an impact on DNA methylation through DNA lesions and base modifications[110]. Specifically, DNA damage resulting from ROS could cause interference in the mechanisms of DNA methylation and lead to global hypomethylation[111]. For example, several studies have indicated that a common DNA lesion induced by ROS (7,8-Dihydro-8-oxoguanine) resulted in abnormal methylation DNA through reduced ability of DNA methyltransferases to successfully perpetuate methylation[112-114]. Another study found that introduction of H_2O_2 into hepatocellular carcinoma cells resulted in the methylation of the promoter of the gene E-cadherin[115]. Although no studies have been conducted examining ROS due to infertility or infertility treatment and epigenetic changes in embryos, it is a plausible pathway linking infertility and epigenetic modification.

All of the above evidence suggests that OS could be a unifying theory of how infertility and infertility treatment could lead to carcinogenesis in children. In summary, DNA damage could be transferred to the embryo through either failure to properly repair sperm DNA fragmentation or abnormal methylation or imprinting. These defects could result in genetic or epigenetic alternations that could ultimately lead to childhood cancer.

Treatment for infertility

There are many additional mechanisms by which treatment for infertility could be linked to childhood cancer in the offspring. One such link is epigenetic disruptions. A growing body of literature suggests an increase in severe disorders related to imprinting after treatment for infertility [3-12]. Changes in imprinting and methylation patterns occur

frequently in cancer formation and could be an early sign of carcinogenesis [22, 23]. If parental treatment for infertility increases methylation and imprinting defects in offspring, this could lead to the formation of childhood cancer. A few different mechanisms have been proposed through which abnormal imprinting could occur in ART procedures; ovarian stimulation, embryo culture medium, and use of sperm from sub-fertile men through ICSI procedures[116-119].

A link between childhood cancer and infertility or its treatment could also be made through associations with congenital abnormalities. Congenital abnormalities may be associated with childhood cancer through a common cause whereby the genetic defect in the abnormality acts as a "hit" in the carcinogenic process. The association between childhood cancer and congenital abnormalities has been well established in many studies[120-122]. The rate of congenital abnormalities is also higher among children born to parents treated for infertility [123-126]as well as in sub-fertile couples who conceived spontanteously[127]. If there is something about infertility treatment (e.g. DNA damage or epigenetic mutations) that causes both congenital abnormalities and childhood cancer then we would expect, given the literature, to observe an increase in childhood cancer in children born to parents with infertility or treated for infertility. However, the increase in childhood cancer may be specific to particular subtypes of childhood cancer and thus more difficult to detect.

Infertility and infertility treatment in infant leukemia and pediatric GCT

While the above biological mechanisms provide potential links between children born after infertility and its treatment and childhood cancer in general, there is also reason to believe that infant leukemia and pediatric GCT might be increased in these children. One possible mechanism for infant leukemia following infertility or its treatment is aberrant DNA methylation. There is some indication that the cell cycle regulatory gene CDKN1C (P57KIP2) has an abnormal methylation pattern in leukemic cells both in adults and children with ALL, although it is more rarely found in childhood ALL[128, 129]. Nonetheless, it is plausible to hypothesize that this gene could be associated with an increase in infant leukemia after infertility treatment. This gene in particular may be important since it is also associated with Beckwith-Wiedemann syndrome, which has been found to be increased in children born after infertility treatment[3, 4, 6-12].

There are also factors associated with infertility or infertility treatment that could increase risk of GCT. Congenital abnormalities of the genital organs have been found to be associated with childhood GCT[62, 63, 67, 69] and studies have indicated increased risk of these abnormalities following ICSI[125, 127]. Another study indicated that there could be an increased risk of cryptorchidism after intrauterine insemination (IUI), although there was no increase after in vitro fertilization (IVF) or ICSI[130]. Thus, the same argument could be made for GCT that was made for childhood cancer in general; that an increase in congenital abnormalities could suggest an increase in cancer. Risk of testicular GCT could also be increased through transmission of infertility to children born through the use of ICSI procedures[131-133]. Male factor infertility has been shown to increase risk for

adult GCT[134-136] and if male children of infertile males are more likely to be infertile themselves, their risk for testicular GCT could also increase. As noted before, however, it is not clear that the link between GCT and male infertility could be extended to childhood GCT, given their histological and cytogenetic differences that imply distinct etiologies.

Conclusions

Epidemiological data on the association between parental infertility and infertility treatment are lacking for most rare childhood cancer diagnoses including infant leukemia and GCT. It is difficult to classify infertility as one disease since there are many potential causes and contributing factors. However, unifying concepts exist for infertility such as increased oxidative stress levels. In addition, treatment for infertility may result in childhood cancer through sperm DNA damage and aberrant imprinting and methylation. Studies of specific subtypes of childhood cancer are needed to fully explore the potential relationship between childhood cancer and parental infertility and its treatment.

Table 1: Studies involving infertility/infertility treatment and childhood leukemia

Type of leukemia	Author	Year	Exposure	Findings
Infant Leukemia	Ross, et al.[a]	2003	Use of clomiphene	Overall: OR=0.37 (0.08-1.80) ALL: OR=0.71 (0.06-8.18) AML: OR=0.31 (0.04-2.73)
Childhood Leukemia: Overall	Roman , et al.	1997	Ever investigated for infertility Ever treated Treated for index child	Ever investigated: OR=2.1 (0.9-4.6) Ever treated: OR=2.1 (0.7-6.4) Treated index: OR=2.0 (0.6-6.9)
	Schuz, et al.	1999	Hormonal treatment for infertility	OR=1.6 (1.0-2.5)
Childhood Leukemia: ALL	Van Steensel-Mol, et al.	1985	Hormone use Consultation for subfertility	Hormone use OR=2.8 (0.7-10.5) Subfertility OR=6.0 (0.9-32.2)
	Wen, et al.[a]	2002	Hormone use	OR=1.0 (0.7-1.6)
	Shu, et al.[a]	2002	Maternal history of infertility, TTP, use of hormones prior to pregnancy	No association found, ORs not reported
	Shaw, et al.	2004	Hormones (fertility or thyroid)	OR=1.0 (0.5-2.0)
Childhood Leukemia: AML	Cnattingius, et al.	1995	History of infertility (> one year involuntary childlessness)	Only one case with treatment (with DS)
Childhood Leukemia: Children with Down syndrome	Puumala, et al.	2007	History of infertility (at least one year involuntary childlessness) TTP for the index child (not trying (reference), ≤ 1 yr, >1 yr)	Infertility history: ALL: OR=0.8 (0.4-1.6) AML: OR=2.2(1.1-4.3) TTP: ALL: OR=1.1 (0.4-2.8) (>1 yr); AML: OR=2.1(0.7-6.1) (>1 yr)

a Based on the same study

Chapter 2

Null associations between infant leukemia and parental infertility or its treatment: a Children's Oncology Group Report

Introduction: Little is known about the potential risk factors for infant leukemia. With the very young age at diagnosis, exposures occurring in the perinatal period are suspected to be causal. Parental infertility and infertility treatment have been studied with regard to childhood cancer in general, but rarely in individual childhood cancer subtypes.

Methods: A case-control study of infant leukemia was conducted through the Children's Oncology Group (COG). The study included cases diagnosed from January 1996 to December 2006 as well as controls selected through random digit dialing and birth certificate tracing. Maternal phone interviews were conducted to obtain information about infertility and infertility treatment as well as demographic factors. All cases as well as subgroups defined by MLL translocation status and leukemia subtype were examined. Statistical analysis was performed using multivariate logistic regression models.

Results: No significant associations between infertility or its treatment and combined infant leukemia were found. In subgroup analysis, there was a significant increase in the risk of MLL- leukemia for children born to women not trying to conceive compared to those trying for less than one year for combined infant leukemia (Odds ratio

(OR) = 1.62, 95% Confidence interval (CI) = 1.01-2.58) and for ALL (OR = 2.50, 95% CI = 1.37-4.57). There was a decrease in the odds of AML for children born to women who reported use of medication to help them become pregnant (OR = 0.40, 95% CI = 0.18-0.93).

Conclusions: There were no positive associations between parental infertility or infertility treatment and infant leukemia. While this was the largest study of infant leukemia to date, selection and recall bias may have impacted the reported associations due to the case-control study design. However, for infant leukemia combined, it appears that we can potentially rule out large increases in risk associated with parental infertility or its treatment.

Introduction

Infant leukemia is a rare subtype of childhood leukemia with an incidence of approximately 40 cases per million infants in the U.S. in 1992-2004 [33]. Leukemia in infants differs from leukemia in older children with variation in type of diagnosis (acute myeloid (AML) and acute lymphoblastic (ALL)), clinical symptoms, tumor genetics, and response to treatment [34]. Given its differences from leukemia in older children, it is necessary to study this subtype separately in order to discover clues that might lead to improved treatment or lower incidence rates. Also, since the relevant exposure(s) most likely occur *in utero*, the study of infant leukemia could help uncover important information about carcinogenesis in general and specifically about carcinogenic exposures before or during pregnancy.

Many studies have been conducted to examine the association between infertility and infertility treatment and childhood cancer. While several large cohort studies have examined assisted reproductive technology (ART) and childhood cancer, few have looked at specific cancer diagnoses. Most studies have found that the total number of cancer cases observed in a cohort of children born after ART were similar to what would be expected [24-29], but have been based on small numbers of cancers overall. Whereas, case-control studies have only examined infertility in general and have found some increases in risk for leukemia, hepatoblastoma, neuroblastoma, and retinoblastoma [30-32]. Rarer diagnoses or subsets of diagnoses have not been frequently examined with respect to infertility or infertility treatment.

Little is known from epidemiological studies about infertility or its treatment as possible risk factors for infant leukemia. One study of leukemia in infants (up to 18 months of age at diagnosis) examined medication recorded in the mother's medical record during pregnancy [54]. This study found a non-significant inverse association with clomiphene, an ovarian stimulant, but the analysis was only based on two cases with reported use. Other studies examining indication or treatment of infertility and childhood leukemia overall have been mixed with some finding an indication of increased risk [31, 55-57] and others finding no association [45, 58-60]. However, all suffered from relatively low power since there were few cases or controls reporting infertility or infertility treatment.

Taken as a whole, the previous literature supports further investigation of infertility and/or infertility treatment and risk of childhood leukemia. Thus, the current study addresses this need and is the first study to look at this exposure within MLL subtypes.

Materials and Methods

Data for this analysis were from a Children's Oncology Group (COG) case-control study of infant leukemia. Cases were collected in two phases for this study. Both phases required cases to have a confirmed diagnosis of acute leukemia prior to 1 year of age. Patients could be diagnosed with either ALL or AML. Cases who died before the study period were eligible for study participation since the main source of data collection was the child's mother. Children were eligible if they were not diagnosed with Down syndrome, had a biological mother who spoke English or Spanish (phase II only), had a

biological mother available by telephone, and were treated or diagnosed at a participating COG institution in the U.S. or Canada. Once cases were identified, the treating physician was contacted and asked to provide permission to contact the child's mother or parental consent to contact was obtained directly. Mothers with physician approval or consent were sent a letter explaining the study and notifying them that they would be contacted by phone. The first phase of recruitment included cases diagnosed between January 1, 1996 and October 13, 2002; the second phase included cases diagnosed between January 1, 2003 and December 31, 2006. In phase I, 348 cases were confirmed eligible from 126 participating COG institutions and 240 of these (69%) completed interviews. In phase II, 345 cases were identified by 133 participating COG institutions as potentially eligible for the study. Of those eligible, 203 (59%) completed interviews.

Control children were selected in two phases for this study coinciding with the case periods. For both phases, controls were required to have a biological mother who spoke English or Spanish (phase II) and was available by telephone. In phase I, controls were selected though random digit dialing (RDD). Numbers were generated using a modification of the methods proposed by Waksberg [137]. Potential phone numbers were generated from case phone numbers at diagnosis. The area code and exchange of the case phone number were retained and the last four digits were randomly selected in order to obtain a control number; facilitating geographic frequency matching. For each number, up to nine contact attempts were performed. If the number resulted in no contact, a refusal, or an ineligible household, subsequent numbers were generated until an eligible control agreed to participate in the study. The mother's name and address

were then obtained along with permission to send a letter. Controls were obtained from 25,516 telephone numbers selected using RDD, of which 11,713 were identified as residential numbers. Using the method outlined by Slattery et al, the RDD household screening response rate was 67% [138]. Maternal telephone interviews were successfully completed for 254 out of 430 potential eligible controls, giving a field response rate of 59% and an overall response rate of 40%.

Phase II controls were selected through state birth registries. Sixteen states that could release birth records and registered a large number of infant leukemia cases in phase I were approached about participation, 15 of which ultimately provided rosters of birth certificate (BC) data. Controls were frequency matched to cases on year of birth and region of residence based on phase I case distribution. The 15 states were allocated to regions to facilitate geographical matching. An introductory letter was sent to 270 potential controls providing information about the study and indicating that an interviewer would contact them by phone. Phone contact was attempted for each potential control successively until an eligible control agreed to participate. In both phases, controls were required to have a biological mother who spoke English or Spanish (phase II) and was available by telephone. Initial contact letters were sent to mothers of 270 children from randomly selected BC of which 267 were found eligible. A total of 70 mothers completed the interview for a total field response rate of 27% (71/267).

Information was collected for cases and controls through maternal interview (see Appendix A for exact wording). The median time from the index child's birth to

interview was 24.9 months for cases and 42.1 months for controls. The maternal interview included questions about pregnancy history, maternal exposures during pregnancy with the participating (index) child, family history of cancer and other diseases, and information about the medical history of the mother. Several questions about infertility and infertility treatment were also asked including length of time to index pregnancy, history of infertility (more than one year of trying without becoming pregnant), history of doctor's visits by mother or index biological father due to non-pregnancy, specific infertility treatment, use of female hormones for ovulation stimulation, and use of female hormones for infertility or conditions related to infertility.

MLL status was determined using the case's file from his or her initial COG institution. Information about molecular or cytogenetic testing for MLL gene rearrangements at the time of diagnosis was collected and reviewed by three independent reviewers. Infants were ultimately classified into three classes: MLL + by molecular or cytogenetic methods, MLL- by molecular or cytogenetic methods, or not enough information to determine MLL status.

The institutional review boards at the University of Minnesota and the participating COG institutions approved this study (see Appendix B for COG institution who approved this study). In addition, health departments for the states providing BC also reviewed and approved this study. All participants provided informed consent prior to participating in the study.

Exposures of interest in this study included maternal age (continuous and in categories <27, 27-34, 35-39, and 40+), history of multiple birth (yes/no), history of

recurrent pregnancy loss (2 or more, 1 or none), time to index pregnancy (not trying, <1 year, ≥1 year), history of infertility (more than one year of trying without becoming pregnant), visit to a doctor by mother or index biological father due to non-pregnancy (yes/no), specific infertility treatment (medication, surgery, or other) (yes/no), use of ovulation stimulating drugs before or during early pregnancy (yes/no). In addition, a composite infertility variable was constructed based on latent class analysis (LCA) including maternal age, history of recurrent pregnancy loss, history of infertility, visit to a doctor by mother or index biological father due to non-pregnancy, and use of ovulation stimulating drugs before or during early pregnancy [139]. This method was used since infertility is difficult to measure and a couple's "true" infertility status is usually unknown. The LCA combines information from many different variables in order to obtain a potentially better measurement of the unknown "true" infertility status. Models with and without maternal age were explored in order to determine if the effect of infertility was only through maternal age or if there was an independent risk factor for infertility apart from age (see Appendix C for more details). LCA was conducted using M-Plus software [140]. Predicted class membership was dichotomized and used as a predictor in a logistic regression model.

Descriptive methods were used to assess the appropriateness of statistical analysis and the functional forms of the relationship between exposures and outcome. Multivariate models were constructed after considering matching variables as well as potential confounders. Exposures were included in the logistic analysis if at least four cases and controls were represented in all exposure categories. Birth year was included

in all analysis as a matching factor. Results are reported as odds ratios (OR) and 95% confidence intervals (CI).

In addition to the overall analysis, subgroups based on subtype (ALL, AML) and by MLL status (MLL+, MLL-) were examined separately. Each subgroup of cases was compared to the entire control set since there was no basis for selecting a subset of control children and using all of the controls maximized power. Model based analysis was performed if there were at least two cases and controls in each exposure category within the subgroup. All logistic regression analysis was performed using SAS 9.1 (SAS Institute, Cary, NC).

Results

A total of 443 cases and 324 controls completed interviews when both phases of recruitment were combined. Descriptive statistics for baseline characteristics are presented in table 1. Case mothers were more likely to be Hispanic, have lower education levels, and have lower household income. Case children were similar to control children with respect to gender, gestational age, and birth weight.

The final selection of confounders included household income as well as maternal race, age, and education. Although geography was a matching variable it did not have an impact on the overall results and was not included in the final models (data not shown).

Multivariate analysis based on all leukemia cases combined as well as by MLL status are presented in table 2. No statistically significant associations were found for

any of the proposed infertility measures and infant leukemia. There was a statistically significant increased risk of MLL- infant leukemia for those who reported not trying to become pregnant compared to those trying for less than one year (OR = 1.62, 95% CI = 1.01-2.58).

Subgroup analyses revealed that this statistically significant association was confined to the ALL MLL- subgroup (OR = 2.50, 95% CI = 1.37-4.57), table 3. Further, there was an increased risk of ALL MLL- infant leukemia in women aged <27 years compared to those aged 27-34 years (OR = 2.03, 95% CI = 1.05-3.91). For the AML subgroup, only one statistically significant association was found; there was a reduced risk of infant leukemia in children born to women who reported taking medication (tablets or injections) to help them become pregnant (OR = 0.40, 95% CI = 0.18-0.93), table 4. While not significant, the OR was similar in women who reported using ovulation stimulating drugs prior to or before knowledge of pregnancy (OR = 0.42, 95% CI = 0.13-1.39).

The LCA classified all of the participants who sought medical advice for non-pregnancy into one group along with one additional participant who indicated use of ovulation stimulating drugs but no medical advice for non-pregnancy. The classification did not change based on whether or not age was included in the model. No associations between the LCA infertility class and infant leukemia were statistically significant (data not shown).

Discussion

We found little evidence of a link between infertility or its treatment and infant leukemia in this study. In fact, some of the statistically significant observed associations were in the opposite direction than hypothesized. Even though the sample size of this study was relatively small, we can generally rule out large positive effects of infertility and infertility treatment on infant leukemia.

The only other study that has examined the association between infertility treatment and infant leukemia specifically, evaluated medication recorded in the mother's medical record during pregnancy and found a non-significant inverse association with clomiphene [54]. In the current study, while no association was found overall, there was an indication of a 58% decrease in the odds of AML with medication used for infertility and a similar, but non-significant OR for use of ovulation stimulating drugs. The inverse association was unexpected. It could be due to a "healthy user" effect, since those who seek out infertility treatments may have better health and dietary intake than women who did not use medical intervention to achieve pregnancy. However, if this were the case we would expect to see this inverse association across all groups examined, which we did not.

We also found an increased risk in the MLL- group both overall and for the ALL MLL- subgroup for women who indicated they were not trying to become pregnant when they conceived the index child that is in the opposite direction of our hypothesis. However, with few significant findings and many comparisons some significant results could be due to chance alone.

There are several strengths to this study. First, this study represents one of the largest case-control studies assembled for infant leukemia. In addition, this is one of the few studies of infant leukemia that has incorporated the presence of MLL translocations into the analyses. Finally, the exposures used in this analysis are specific to infertility or infertility treatment and, as such, can better evaluate the relationship between this exposure and infant leukemia.

There are also several limitations to this study. First, as it uses the case-control design it is subject to recall bias. Case-control studies are uniquely prone to recall bias, which occurs when cases recall past exposures differently from controls, leading to an OR that reflects a difference between the two groups based on recall rather than an actual difference in exposure [141]. Several studies have assessed the magnitude of maternal recall bias and have found varying levels, which were dependent upon the exposure studied [142, 143]. One study on malformations that examined history of infertility found that women reported malformations much less than what was reported in medical records. Cases in this study were slightly more likely to report a history of infertility than controls [143]. Another study showed little difference in the sensitivity of reporting previous spontaneous abortion in cases and controls in a study on sudden infant death syndrome [142]. Assessments of the validity of time to pregnancy have been mixed with some indicating good validity[144-146] with another suggesting lower validity particularly in women with a longer time to pregnancy[147]. We do note that the young age at diagnosis allowed information on exposures during pregnancy to be assessed after only a short period of time had passed. This short period may help to minimize errors in recall.

Selection bias occurs in a case-control study when the probability of participation or selection into the study is different based on case-control status and the underlying exposure of interest [148]. An analysis of potential selection bias in our study found that controls differed from the underlying population of interest in terms of maternal age, maternal education level, birth weight, gestational age, race, and marital status [149]. In the current analysis, maternal age, education, and income were found to be confounders of the association between infertility measures and infant leukemia. Since these factors are also related to selection and are antecedent to the exposure and disease, selection bias due to these factors should be mitigated in the adjusted analysis [150].

Another potential limitation was the recruitment of controls in two different time periods by two different methods. Controls could be heterogeneous and this could have led to problems in estimating an overall effect by combining the two study phases. However, in a study examining possible differences between the two control populations, the controls from each time period were found not to differ significantly from one another except for reported smoking during pregnancy [149]. The difference in smoking rates could be due to a temporal trend since reported smoking during pregnancy appears to be declining over time [151, 152]. Thus, it was necessary to include controls from both study phases so that differences between cases and controls would not be influenced by temporal trends.

Finally, even though this study was large comparatively, the sample size was still small in absolute terms, which may have resulted in imprecisely estimated parameters. Also, with several exposure variables of interest and multiple subgroup analyses, our

results are likely to include some false positives. Taken together, our results must be interpreted with caution and considered exploratory.

We found little evidence of an association between infertility and infertility treatment and infant leukemia in the largest study of infant leukemia yet conducted. Although there are several limitations both of the study itself and the study design used, it is unlikely that a strong association was missed.

Table 1: Demographic factors

	Controls n (%)	Cases n (%)	OR[a]	95% CI
Maternal characteristics				
Race/Ethnicity				
White	273 (84.5)	334 (75.6)	Ref	
Black	18 (5.6)	18 (4.1)	0.65	0.31-1.33
Hispanic	15 (4.6)	55 (12.4)	2.62	1.42-4.83
Other	17 (5.3)	35 (7.9)	1.66	0.88-3.11
Education				
≤ High school	91 (28.2)	149 (33.7)	1.46	1.00-2.13
Some post HS	112 (34.7)	125 (28.3)	Ref	
College graduate	120 (37.2)	168 (38.0)	1.10	0.76-1.58
Household income				
≤ $30,000	95 (29.6)	157 (35.8)	Ref	
$30,001-$75,000	145 (45.2)	189 (43.1)	0.80	0.56-1.13
> $75,000	81 (25.2)	93 (21.2)	0.56	0.37-0.84
Child characteristics				
Gender				
Male	156 (48.1)	218 (49.2)	Ref	
Female	168 (51.9)	225 (50.8)	0.97	0.72-1.31
Gestational age (weeks)				
<37	24 (7.4)	32 (7.2)	0.79	0.44-1.43
37- 40	259 (79.9)	360 (81.3)	Ref	
≥ 41	41 (12.7)	51 (11.5)	1.03	0.65-1.62
Birth weight (grams)				
Mean (sd)	3436.33 (591.76)	3477.26 (572.45)	1.12[b]	0.98-1.28

a Logistic regression models were adjusted for year of birth (quartiles)
b Per 500 gram increase

Table 2: Association between infant leukemia and infertility and infertility treatment

	Controls n (%)	Cases n (%)	OR[a]	95% CI	MLL+ n(%)	OR[a]	95% CI	MLL- n(%)	OR[a]	95% CI
Prior fetal loss										
None	241 (74.4)	337 (76.1)	Ref		171 (75.0)	Ref		114 (78.1)	Ref	
One	64 (19.8)	76 (17.2)	1.11	0.74-1.67	42 (18.4)	1.31	0.80-2.15	22 (15.1)	0.89	0.50-1.57
Two or more	19 (5.9)	30 (6.8)	1.39	0.73-2.65	15 (6.6)	1.57	0.71-3.44	10 (6.8)	1.44	0.61-3.42
History of multiple birth[b]										
None	315 (97.2)	435 (98.2)	Ref		225 (98.7)	Ref		143 (97.9)	Ref	
At least one multiple birth	9 (2.8)	8 (1.8)	0.78	0.27-2.25	3 (1.3)	0.51	0.11-2.33	3 (2.1)	0.79	0.19-3.23
Maternal age (mean (SD))[c]	29.8 (5.4)	29.1 (5.8)	0.99	0.96-1.02	28.8 (5.5)	0.98	0.94-1.02	29.0 (6.0)	0.99	0.95-1.03
Maternal age (categorical)										
< 27	97 (30.0)	158 (35.7)	1.15	0.78-1.68	83 (36.6)	1.16	0.73-1.85	56 (38.4)	1.41	0.84-2.36
27-34	168 (52.0)	214 (48.4)	Ref		113 (49.9)	Ref		63 (43.2)	Ref	
35-39	47 (14.6)	61 (13.8)	1.06	0.66-1.68	29 (12.8)	1.02	0.57-1.83	21 (14.4)	1.20	0.64-2.26
≥40	11 (3.4)	9 (2.0)	0.73	0.28-1.86	2 (0.9)	0.43	0.09-2.05	6 (4.1)	1.70	0.58-4.97
Use of ovarian stimulating drugs										
No	309 (95.4)	424 (95.7)	Ref		218 (95.6)	Ref		140 (95.9)	Ref	
Yes	15 (4.6)	19 (4.3)	1.01	0.48-2.13	10 (4.4)	0.84	0.33-2.15	6 (4.1)	0.96	0.35-2.66
Use of hormones for infertility										
No	312 (96.3)	422 (95.3)	Ref		215 (94.3)	Ref		140 (95.9)	Ref	
Yes	12 (3.7)	21 (4.7)	1.37	0.62-3.02	13 (5.7)	1.45	0.57-3.71	6 (4.1)	1.39	0.48-4.01
Ever try for one year or more										
No	248 (76.5)	345 (78.1)	Ref		174 (76.7)	Ref		118 (80.8)	Ref	
Yes	76 (23.5)	97 (21.9)	1.12	0.77-1.63	53 (23.3)	1.23	0.78-1.94	28 (19.2)	1.01	0.59-1.72
Ever visit a doctor or clinic for non-pregnancy										
No	267 (82.4)	372 (84.0)	Ref		192 (84.2)	Ref		121 (82.9)	Ref	
Yes	57 (17.6)	71 (16.0)	0.99	0.64-1.51	36 (15.8)	0.97	0.58-1.64	25 (17.1)	1.15	0.65-2.05
Surgical treatment[d]										
No	308 (95.1)	427 (96.4)	Ref		220 (96.5)	Ref		138 (94.5)	Ref	
Yes	16 (4.9)	16 (3.6)	0.79	0.37-1.72	8 (3.5)	0.69	0.26-1.83	8 (5.5)	1.40	0.55-3.56

Medication[d]										
No	292 (90.1)	407 (91.9)	Ref		208 (91.2)	Ref		134 (91.8)	Ref	
Yes	32 (9.9)	36 (8.1)	0.85	0.49-1.47	20 (8.8)	0.83	0.43-1.62	12 (8.2)	0.94	0.44-2.01
Other treatment[d]										
No	312 (96.3)	426 (96.2)	Ref		219 (96.1)	Ref		139 (95.2)	Ref	
Yes	12 (3.7)	17 (3.8)	1.02	0.45-2.33	9 (3.9)	1.05	0.38-2.91	7 (4.8)	1.60	0.57-4.46
Time to index pregnancy										
Not trying	108 (33.4)	170 (38.4)	1.22	0.86-1.72	77 (33.8)	0.98	0.63-1.53	66 (45.2)	1.62	1.01-2.58
Less than one year of trying	175 (54.2)	227 (51.2)	Ref		125 (54.8)	Ref		67 (45.9)	Ref	
One year or more of trying	40 (12.4)	46 (10.4)	1.08	0.65-1.79	26 (11.4)	1.10	0.60-2.01	13 (8.9)	0.96	0.46-2.00

a Logistic regression models were adjusted for year of birth (quartiles), maternal age (continuous), maternal education, maternal race, and household income
b Prior to index pregnancy
c OR for one year increase in age
d Assumed no if no visit to a clinic or doctor for non-pregnancy

Table 3: Association between infant ALL and infertility and infertility treatment

	Controls n (%)	ALL n (%)	OR[a]	95% CI	ALL/MLL+ n (%)	OR[a]	95% CI	ALL/MLL- n (%)	OR[a]	95% CI
Prior fetal loss										
None	241 (74.4)	199 (75.4)	Ref		116 (73.9)	Ref		61 (79.2)	Ref	
One	64 (19.8)	44 (16.7)	1.14	0.72-1.82	29 (18.5)	1.44	0.83-2.49	9 (11.7)	0.64	0.29-1.42
Two or more	19 (5.9)	21 (8.0)	1.80	0.89-3.65	12 (7.6)	1.87	0.80-4.34	7 (9.1)	2.18	0.80-5.92
History of multiple birth[b]										
None	315 (97.2)	258 (97.7)	Ref		154 (98.1)	Ref		75 (97.4)	Ref	
At least one multiple birth	9 (2.8)	6 (2.3)	1.12	0.36-3.51	3 (1.9)	0.78	0.17-3.54	2 (2.6)	1.09	0.22-5.44
Maternal age (mean (SD))[c]	29.8 (5.4)	28.7 (5.6)	0.98	0.95-1.02	28.7 (5.5)	0.99	0.94-1.03	28.7 (6.0)	0.97	0.91-1.02
Maternal age (categorical)										
< 27	97 (30.0)	102 (38.6)	1.24	0.81-1.90	59 (37.6)	1.12	0.67-1.87	33 (42.9)	2.03	1.05-3.91
27-34	168 (52.0)	126 (47.7)	Ref		78 (49.7)	Ref		30 (39.0)	Ref	
35-39	47 (14.6)	29 (11.0)	0.84	0.48-1.47	18 (11.5)	0.96	0.49-1.86	9 (11.7)	0.99	0.42-2.34
≥40	11 (3.4)	7 (2.7)	1.00	0.36-2.76	2 (1.3)	0.63	0.13-3.00	5 (6.5)	2.61	0.80-8.58
Use of ovarian stimulating drugs										
No	309 (95.4)	250 (94.7)	Ref		150 (95.5)	Ref		73 (94.8)	Ref	
Yes	15 (4.6)	14 (5.3)	1.42	0.64-3.16	7 (4.5)	1.06	0.39-2.89	4 (5.2)	1.20	0.37-3.93
Use of hormones for infertility										
No	312 (96.3)	251 (95.1)	Ref		150 (95.5)	Ref		73 (94.8)	Ref	
Yes	12 (3.7)	13 (4.9)	1.73	0.73-4.11	7 (4.5)	1.50	0.52-4.35	4 (5.2)	1.63	0.47-5.60
Ever try for one year or more										
No	248 (76.5)	197 (74.9)	Ref		117 (75.0)	Ref		59 (76.6)	Ref	
Yes	76 (23.5)	66 (25.1)	1.36	0.89-2.07	39 (25.0)	1.34	0.81-2.22	18 (23.4)	1.36	0.70-2.62
Ever visit a doctor or clinic for non-pregnancy										
No	267 (82.4)	217 (82.2)	Ref		131 (83.4)	Ref		62 (80.5)	Ref	
Yes	57 (17.6)	47 (17.8)	1.29	0.80-2.08	26 (16.6)	1.20	0.68-2.12	15 (19.5)	1.40	0.69-2.83
Surgical treatment[d]										
No	308 (95.1)	254 (96.2)	Ref		152 (96.8)	Ref		72 (93.5)	Ref	
Yes	16 (4.9)	10 (3.8)	0.86	0.36-2.08	5 (3.2)	0.67	0.22-2.08	5 (6.5)	1.51	0.50-4.61

Medication[d]										
No	292 (90.1)	238 (90.2)	Ref		143 (91.1)	Ref		68 (88.3)	Ref	
Yes	32 (9.9)	26 (9.8)	1.26	0.70-2.29	14 (8.9)	1.04	0.50-2.14	9 (11.7)	1.55	0.65-3.69
Other treatment[d]										
No	312 (96.3)	254 (96.2)	Ref		151 (96.2)	Ref		73 (94.8)	Ref	
Yes	12 (3.7)	10 (3.8)	1.14	0.45-2.86	6 (3.8)	1.30	0.44-3.88	4 (5.2)	1.67	0.48-5.81
Time to index pregnancy										
Not trying	108 (33.4)	104 (39.4)	1.32	0.89-1.96	53 (33.8)	1.02	0.62-1.65	38 (49.4)	2.50	1.37-4.57
Less than one year of trying	175 (54.2)	127 (48.1)	Ref		85 (54.1)	Ref		29 (37.7)	Ref	
One year or more of trying	40 (12.4)	33 (12.5)	1.31	0.75-2.28	19 (12.1)	1.08	0.55-2.12	10 (13.0)	1.88	0.80-4.42

a Logistic regression models were adjusted for year of birth (quartiles), maternal age (continuous), maternal education, maternal race, and household income
b Prior to index pregnancy
c OR for one year increase in age
d Assumed no if no visit to a clinic or doctor for non-pregnancy

Table 4: Association between infant AML and infertility and infertility treatment

	Controls n (%)	AML n (%)	OR[a]	95% CI	AML/MLL+ n (%)	OR[a]	95% CI	AML/MLL- n (%)	OR[a]	95% CI
Prior fetal loss										
None	241 (74.4)	133 (77.3)	Ref		54 (79.4)	Ref		50 (75.8)	Ref	
One	64 (19.8)	31 (18.0)	1.04	0.61-1.76	12 (17.6)	1.02	0.46-2.26	13 (19.7)	1.17	0.57-2.41
Two or more	19 (5.9)	8 (4.7)	0.86	0.33-2.22	2 (2.9)	0.80	0.16-4.00	3 (4.5)	0.89	0.23-3.40
History of multiple birth[b]										
None	315 (97.2)	170 (98.8)	Ref		68 (100.0)	Ref		65 (98.5)	Ref	
At least one multiple birth	9 (2.8)	2 (1.2)	0.34	0.06-1.92	0 (0.0)	**		1 (1.5)	**	
Maternal age (mean (SD))[c]	29.8 (5.4)	29.7 (5.9)	1.01	0.97-1.05	28.9 (5.6)	0.96	0.89-1.02	29.5 (6.0)	1.02	0.96-1.08
Maternal age (categorical)										
< 27	97 (30.0)	52 (30.4)	0.97	0.58-1.61	23 (34.3)	1.46	0.68-3.15	21 (31.8)	0.86	0.42-1.76
27-34	168 (52.0)	87 (50.9)	Ref		35 (52.2)	Ref		32 (48.5)	Ref	
35-39	47 (14.6)	30 (17.5)	1.50	0.84-2.69	9 (13.4)	0.95	0.38-2.39	12 (18.2)	1.36	0.64-2.92
≥40	11 (3.4)	2 (1.2)	0.47	0.10-2.22	0 (0.0)	**		1 (1.5)	**	
Use of ovarian stimulating drugs										
No	309 (95.4)	167 (97.1)	Ref		65 (95.6)	Ref		64 (97.0)	Ref	
Yes	15 (4.6)	5 (2.9)	0.42	0.13-1.39	3 (4.4)	0.46	0.09-2.37	2 (3.0)	0.64	0.13-3.10
Use of hormones for infertility										
No	312 (96.3)	164 (95.3)	Ref		62 (91.2)	Ref		64 (97.0)	Ref	
Yes	12 (3.7)	8 (4.7)	1.01	0.35-2.88	6 (8.8)	1.86	0.52-6.71	2 (3.0)	1.05	0.21-5.20
Ever try for one year or more										
No	248 (76.5)	143 (83.1)	Ref		56 (82.4)	Ref		56 (84.8)	Ref	
Yes	76 (23.5)	29 (16.9)	0.80	0.47-1.34	12 (17.6)	0.99	0.46-2.14	10 (15.2)	0.71	0.33-1.55
Ever visit a doctor or clinic for non-pregnancy										
No	267 (82.4)	149 (86.6)	Ref		59 (86.8)	Ref		56 (84.8)	Ref	
Yes	57 (17.6)	23 (13.4)	0.66	0.37-1.19	9 (13.2)	0.67	0.28-1.62	10 (15.2)	0.96	0.43-2.14
Surgical treatment[d]										
No	308 (95.1)	166 (96.5)	Ref		65 (95.6)	Ref		63 (95.5)	Ref	
Yes	16 (4.9)	6 (3.5)	0.74	0.26-2.13	3 (4.4)	0.84	0.20-3.46	3 (4.5)	1.12	0.29-4.40

Medication[d]										
No	292 (90.1)	162 (94.2)	Ref		62 (91.2)	Ref		63 (95.5)	Ref	
Yes	32 (9.9)	10 (5.8)	0.40	0.18-0.93	6 (8.8)	0.59	0.20-1.76	3 (4.5)	0.41	0.11-1.48
Other treatment[d]										
No	312 (96.3)	165 (95.9)	Ref		65 (95.6)	Ref		63 (95.5)	Ref	
Yes	12 (3.7)	7 (4.1)	1.06	0.36-3.14	3 (4.4)	1.02	0.19-5.45	3 (4.5)	1.68	0.42-6.76
Time to index pregnancy										
Not trying	108 (33.4)	65 (37.8)	1.21	0.77-1.92	24 (35.3)	1.20	0.59-2.43	27 (40.9)	1.19	0.63-2.24
Less than one year of trying	175 (54.2)	94 (54.7)	Ref		37 (54.4)	Ref		36 (54.5)	Ref	
One year or more of trying	40 (12.4)	13 (7.6)	0.75	0.36-1.55	7 (10.3)	1.32	0.49-3.52	3 (4.5)	0.37	0.10-1.32

a Logistic regression models were adjusted for year of birth (quartiles), maternal age (continuous), maternal education, maternal race, and household income
b Prior to index pregnancy
c OR for one year increase in age
d Assumed no if no visit to a clinic or doctor for non-pregnancy

Chapter 3

Pediatric GCT and parental infertility and infertility treatment: a Children's Oncology Group Report

Introduction: Few risk factors have been established for childhood germ cell tumors (GCT). Parental infertility and infertility treatment may be associated with GCT development but these risk factors have not been fully investigated.

Methods: A case-control study of childhood GCT was conducted through the Children's Oncology Group (COG). Cases, under the age of 15 at diagnosis, were recruited through COG institutions from January 1993 to December 2002. Controls were obtained through random digit dialing. Information about infertility and infertility treatment along with demographic factors was collection through maternal interviews. Subgroups created by gender, age at diagnosis, and tumor location were examined separately. Statistical analysis was performed using multivariate logistic regression models.

Results: Overall, no association between GCT and infertility or its treatment was found. In subgroup analysis, females whose mothers had two or more fetal losses were found to be at increased risk for non-gonadal tumors (Odds ratio (OR) = 3.32, 95% Confidence interval (CI) = 1.12-9.88). Younger maternal age was associated with a lower risk of gonadal GCT in females (OR = 0.52, 95% CI = 0.28-0.96). There was an

increased risk of all GCT and gonadal GCT in males for those born to older mothers (OR = 2.88, 95% CI = 1.13-7.37 and OR = 3.70, 95% CI = 1.12-12.24).

Conclusion: While no association between parental infertility or its treatment and childhood GCT was found overall, possible associations with maternal age and history of recurrent fetal loss were found in subgroups defined by gender.

Introduction

Childhood germ cell tumors (GCT) are a group of heterogeneous diagnoses with a common origin in the primordial germ cell[153]. There is a distinctive U-shaped incidence curve by age with an incidence rates of 21.0, 3.9, 2.2, 7.4, and 29.0 per million for age groups <1, 1-4, 5-9, 10-14, and 15-19 respectively in the United States from 1992-2004[153]. In addition to the wide variance with age, there is a difference in the location of the tumors by sex. In females below the age of 4, the most common type of GCT is non-gonadal GCT whereas in boys testicular GCT and non-gonadal GCT have approximately equal incidence rates in infancy and, after that, gonadal GCT predominates until age four[153]. Incidence is low for both males and females until around puberty when the incidence of ovarian GCT in females and testicular GCT in males rises dramatically[153].

The literature on the etiology of GCT in children under the age of 15 is sparse, and associations with potential risk factors have been noted only inconsistently[62-67, 71, 73]. Parental infertility and infertility treatment have not been studied previously in pediatric GCT; however, there are reasons that an association would be plausible. For example, superovulation, as seen with exogenous hormones, has been shown to disrupt acquisition and maintenance of genomic imprinting in developing embryos[154]. This abnormal imprinting could potentially lead to tumor development. Another potential link is that male infertility has been shown to be a risk factor for adult GCT[135] and has also been shown to be transmittable to the next generation through the use of intracytoplasmic

sperm injection procedures[131-133]. Male children of infertile males may be more likely to be infertile themselves, increasing their risk for testicular GCT.

We used data from a Children's Oncology Group (COG) case-control study to evaluate the potential association between parental infertility and its treatment and GCT in children.

Materials and Methods

The methods for this study have been detailed previously[65, 66]. Briefly, a total of 84 member institutions of the COG provided cases for this study. Cases were eligible if they had a newly diagnosed GCT under the age of 15 from January 1993 to December of 2002. Specific GCT diagnoses included germinoma, embryonal carcinoma, yolk sac tumor, choriocarcinoma, immature teratoma, and mixed GCT. Patients with tumors located in the brain were excluded. Cases were required to have a biological mother available who spoke English and have a telephone in their residence. The treating physician had to give approval in order for study coordinators to contact the case's mother.

Controls were selected through random digit dialing and were frequency matched to cases based on sex, age, and telephone exchange. Using methods similar to those of Waksberg [155], potential control phone numbers were generated from case phone numbers. The area code and exchange of the case phone number were retained and the last four digits were randomly selected in order to obtain a control number. If a residential number was reached screening questions were asked to determine whether or not a child under the age of 15 was present in the household. Case-control ratios were

different based on sex; for males the ratio was 1:2 and females 1:1 since GCT in children is more common in females. Approval for this study was granted by the institutional review boards at each participating COG institution as well as from the University of Minnesota where this analysis was performed (see Appendix B for COG institutions that approved this study).

Information on potential exposures was collected for cases and controls through maternal interview (see Appendix A for study forms). The interview included questions about pregnancy history, maternal exposures during pregnancy with the index child, family history of cancer and other diseases, and information about the medical history of the mother. In the maternal questionnaire, several questions about infertility and infertility treatment were asked including length of time to index pregnancy, history of infertility (more than one year of trying without becoming pregnant), visit to a doctor by mother or index biological father due to non-pregnancy, specific infertility treatment, and use of female hormones such as fertility drugs or birth controls pills in the year before or during pregnancy.

Unconditional logistic regression was used to explore the association between infertility and infertility treatment and GCT. Exposures were included in the logistic analysis if at least four cases and controls were represented in all exposure categories. Matching factors included child's sex and year of birth, so these variables were included in all analyses. Although geography was also frequency matched, data was not available to adjust for this in the analysis. In addition potential confounding variables (maternal age, maternal race, household income, maternal education, and child's gestational age)

were included in the models. Results are reported as odds ratios (OR) and 95% confidence intervals (CI). Subgroup analysis was performed for age at diagnosis (<5, 5+) and tumor location (non-gonadal, gonadal), and gender. Gender was analyzed in subgroups rather than through interactions since the etiology of GCT may differ by gender. All analysis was performed using SAS 9.1 (SAS institute, Inc, Cary, NC).

An additional method for assessing infertility was explored based on latent class analysis (LCA)[139]. The variables used in the latent class analysis were maternal age, history of infertility, use of female hormones before or during pregnancy, history of multiple birth, and history of recurrent pregnancy loss. While each variable individually has the potential for misclassification of the true exposure of interest, using all variables together may mitigate this misclassification. Models with and without maternal age were explored in order to determine if the effect of infertility was only through maternal age or if there was an independent risk factor for infertility apart from age. LCA was conducted using M-Plus software[140]. Predicted class membership was dichotomized and used as a predictor in a logistic regression model along with potential confounders (see Appendix C for more details).

Results

Out of 344 eligible cases, 278 completed the maternal interview. Controls were obtained from a total of 17,292 randomly selected phone numbers with 5,912 residential numbers identified. There were 634 households with confirmed eligible children, of which 422 completed maternal interviews (67%).

Demographic characteristics of cases and controls are presented in table 1. Case mothers were more likely to be non-white and have a high school education or less. Case and control children were similar with regard to gestational age and birth weight.

Overall results suggested little relationship between infertility or infertility treatment and GCT (table 2). Gonadal and non-gonadal subgroup results were fairly concordant with the overall results. However, analysis for each gender separately indicated some potential differences between cases and controls (tables 3 and 4). For females, there was a marginally increased risk of GCT in children born to women with two or more fetal losses (OR = 2.12, 95% CI = 0.84-5.36), in the non-gonadal group this finding reached statistical significance (OR = 3.32, 95% CI = 1.12-9.88). Maternal age <27, when compared to mothers aged 27-34, was found to be marginally associated with a lower risk of GCT in females overall (OR = 0.67, 95% CI = 0.43-1.05) and within gonadal GCT (OR = 0.52, 95% CI = 0.28-0.96). A non-significant lower risk with higher maternal age was also seen in the female subgroup both overall and for gonadal tumors. Finally, in males there appeared to be an increased risk of GCT in children born to mothers aged 35 years or greater, compared to mothers aged 27.34, both overall (OR = 2.88, 95% CI = 1.13-7.37) and in gonadal tumors (OR = 3.70, 95% CI = 1.12-12.24). In younger mothers, <27 years of age, there was also a trend towards increased risk of male GCT overall and in gonadal tumors although this did not reach statistical significance.

No notable associations were observed in analysis stratified by age at diagnosis (data not shown).

Discussion

While parental infertility or treatment was not found to be a risk factor for childhood GCT overall, there were some associations in clinically relevant subgroups. Specifically, we found that history of recurrent pregnancy loss elevated risk of non-gonadal GCT in females. Although the American Society for Reproductive Medicine defines recurrent pregnancy loss as a separate disease than infertility[156], other evidence shows that repeated pregnancy loss may, in fact, be related to infertility, particularly in the male[157]. Still, there are many potential causes of recurrent pregnancy loss including a large percentage with no known cause[158]; thus possible underlying reasons for this association would be difficult to determine. However, the association found in this study could represent a novel risk factor for non-gonadal GCT in females that may warrant further examination.

The associations with maternal age in female and male gonadal GCT are in opposite directions with an apparent decrease in risk for females born to younger and older mothers and an apparent increase in risk for males born to younger and older mothers when compared to our reference group (maternal age 27-34 years). This type of qualitative interaction is more likely to be due to chance than represent a real biological phenomenon since it seems unlikely that the effect of maternal age would be opposite for male and female gonadal GCT. As others have noted, it is generally implausible biologically for a single exposure to both cause and protect from the same disease in different subgroups, but is more likely a statistical anomaly[159]. In addition there is little

evidence for a maternal age effect in GCT both in childhood[160] and in adolescent and adult testicular cancer[161].

Very few studies of pediatric GCT have been conducted. Of those that have been carried out, this study includes the largest number of cases. This relatively large size allowed us to examine subgroups within GCT that potentially have different etiologies and is the study's greatest strength.

Several limitations also apply to this study. First, even though the number of GCT cases was relatively large it was still small in absolute number, particularly in subgroup analysis, leaving us with wide confidence intervals and limited ability to detect moderate associations. Many associations were examined in this study as well, which could lead to false positive findings due to chance alone. There is also the potential for selection bias to influence the study results. Our controls were more likely to be white and have higher income and education compared to cases. These differences may indicate some level of selection bias. However, since these factors are antecedents to both exposure and diseases, adjusting for them in the model may be expected to reduce selection bias[150].

Selection of cases through COG may also have introduced bias, as the proportion of children with cancer seen at COG institutions decreases as the age of the child increases[162]. However, most cases of childhood GCT occur before the age of 5 so that COG institutions would be likely to see a majority of cases; in the current study over half of the cases in this study were diagnosed at 5 years or younger. In addition, a previous analysis found that a higher proportion of girls with GCT participated as well as a higher

percentage of children who were white[65]. This might indicate some differential selection in the cases; however, since controls are also expected to have a higher proportion of children with white race, this could, in fact, mitigate the impact of selection bias. In any case, COG is the only organization that could practicably facilitate case recruitment for such a rare disease in North America.

A final limitation is the potential for recall bias since the exposures of interest could have taken place many years prior to the study. Other studies have examined the possibility of recall bias relating to infertility and infertility treatment. History of infertility was found to be self-reported less often than on medical records as well as slightly more often by cases than controls in one study[143]. Another study showed little difference in the sensitivity of reporting previous spontaneous abortion in cases and controls in a study on sudden infant death syndrome[142]. In this study, the only significant associations were found for maternal age and history of recurrent fetal loss both of which appear to be less subject to recall bias.

While we did not find any association with infertility or infertility treatment and pediatric germ cell tumors, we did find some possible associations with maternal age and recurrent pregnancy loss in sex and location specific subgroups. These associations could be due to chance but should be considered in future studies.

Table 1: Demographic characteristics

	Controls n (%)	Cases n (%)
Mother's race		
White	356 (84.4)	213 (76.9)
Non-white	66 (15.6)	64 (23.1)
Missing		
Mother's education		
≤ High school	166 (39.3)	138 (49.8)
Some post HS	97 (23.0)	53 (19.1)
College graduate	159 (37.7)	86 (31.0)
Missing		
Household income		
≤ $30,000	192 (45.9)	145 (52.9)
$30,001-$50,000	124 (29.7)	63 (23.0)
> $50,000	102 (24.4)	66 (24.1)
Missing		
Index child's gestational age		
< 37 weeks	44 (10.4)	35 (12.6)
37-41 weeks	343 (81.1)	220 (79.1)
≥ 42 weeks	36 (8.5)	23 (8.3)
Index child's birth weight Mean (SD)	3373.72 (590.31)	3354.27 (680.58)
Index child's gender[c]		
Male	181 (42.9)	83 (29.9)
Female	241 (57.1)	195 (70.1)
Missing		
Index child's age at diagnosis		
<1 years		57 (20.5)
1-4 years		91 (32.7)
5-9 years		41 (14.7)
10-15 years		89 (32.0)
Type of tumor		
Gonadal		144 (53.1)
Non-gonadal		120 (44.3)
Metastatic		7 (2.6)

Table 2: Association between childhood GCT and infertility and infertility treatment

	Controls n (%)	Cases n (%)	OR[a]	95% CI	Gonadal n (%)	OR[a]	95% CI	Non-gonadal n (%)	OR[a]	95% CI
Prior fetal loss										
None	332 (78.5)	227 (81.7)	Ref		119 (82.6)	Ref		97 (80.8)	Ref	
One	74 (17.5)	34 (12.2)	0.65	0.41-1.03	19 (13.2)	0.65	0.37-1.16	13 (10.8)	0.57	0.29-1.12
Two or more	17 (4.0)	17 (6.1)	1.30	0.63-2.71	6 (4.2)	0.96	0.35-2.63	10 (8.3)	1.51	0.62-3.68
History of multiple birth										
None	410 (96.9)	269 (96.8)	Ref		139 (96.5)	Ref		117 (97.5)	Ref	
At least one multiple birth	13 (3.1)	9 (3.2)	1.08	0.43-2.67	5 (3.5)	1.29	0.43-3.91	3 (2.5)	[d]	
Maternal age										
< 27	187 (44.2)	126 (45.3)	0.84	0.59-1.21	64 (44.4)	0.79	0.50-1.25	54 (45.0)	0.91	0.55-1.50
27-34	197 (46.6)	123 (44.2)	Ref		66 (45.8)	Ref		54 (45.0)	Ref	
≥35	39 (9.2)	29 (10.4)	1.23	0.70-2.15	14 (9.7)	1.23	0.61-2.49	12 (10.0)	1.05	0.49-2.29
Use of OS drugs[b]										
No	410 (97.4)	273 (98.2)	Ref		139 (96.5)	Ref		120 (100.0)	Ref	
Yes	11 (2.6)	5 (1.8)	0.84	0.28-2.58	5 (3.5)	1.48	0.48-4.63	0 (0.0)	[d]	
Ever try for one year or more										
No	334 (79.1)	221 (79.5)	Ref		112 (77.8)	Ref		96 (80.0)	Ref	
Yes	88 (20.9)	57 (20.5)	0.95	0.64-1.41	32 (22.2)	1.01	0.62-1.63	24 (20.0)	0.91	0.52-1.57
Time to index pregnancy										
Not trying	191 (45.3)	134 (48.4)	0.93	0.65-1.32	68 (47.2)	0.97	0.63-1.51	58 (48.7)	0.92	0.56-1.52
Less than one year of trying	183 (43.4)	119 (43.0)	Ref		61 (42.4)	Ref		52 (43.7)	Ref	
One year or more of trying	48 (11.4)	24 (8.7)	0.71	0.40-1.26	15 (10.4)	0.86	0.44-1.72	9 (7.6)	0.65	0.29-1.49
LCA infertility[c] (w/mat age)										
No	390 (92.2)	254 (91.4)	Ref		130 (90.3)	Ref		111 (92.5)	Ref	
Yes	33 (7.8)	24 (8.6)	1.05	0.56-1.98	14 (9.7)	1.32	0.62-2.79	9 (7.5)	0.78	0.32-1.89
LCA infertility[c] (w/o mat age)										
No	408 (96.5)	267 (96.0)	Ref		138 (95.8)	Ref		115 (95.8)	Ref	
Yes	15 (3.5)	11 (4.0)	1.18	0.51-2.73	6 (4.2)	1.20	0.43-3.32	5 (4.2)	1.23	0.39-3.86

a Logistic regression models were adjusted for child's age, sex, and gestational age as well as maternal age, race, education, and household income
b Prior to or before knowledge or pregnancy
c Infertility predicted from the latent class model
d Sample size too small for logistic regression analysis

Table 3: Association between childhood GCT and infertility and infertility treatment (Females only)

	Controls n (%)	Cases n (%)	OR[a]	95% CI	Gonadal n (%)	OR[a]	95% CI	Non-gonadal n (%)	OR[a]	95% CI
Prior fetal loss										
None	185 (76.8)	157 (80.5)	Ref		80 (82.5)	Ref		69 (77.5)	Ref	
One	48 (19.9)	23 (11.8)	0.59	0.34-1.03	12 (12.4)	0.48	0.22-1.02	10 (11.2)	0.61	0.28-1.36
Two or more	8 (3.3)	15 (7.7)	2.12	0.84-5.36	5 (5.2)	0.77	0.21-2.87	10 (11.2)	3.32	1.12-9.88
History of multiple birth										
None	235 (97.5)	187 (95.9)	Ref		93 (95.9)	Ref		86 (96.6)	Ref	
At least one multiple birth	6 (2.5)	8 (4.1)	1.65	0.53-5.15	4 (4.1)	1.55	0.36-6.61	3 (3.4)	[d]	
Maternal age										
< 27	119 (49.4)	90 (46.2)	0.67	0.43-1.05	44 (45.4)	0.52	0.28-0.96	41 (46.1)	0.87	0.48-1.58
27-34	97 (40.2)	88 (45.1)	Ref		46 (47.4)	Ref		39 (43.8)	Ref	
≥35	25 (10.4)	17 (8.7)	0.79	0.39-1.61	7 (7.2)	0.63	0.22-1.76	9 (10.1)	0.87	0.35-2.20
Use of OS drugs[b]										
No	234 (97.9)	191 (97.9)	Ref		93 (95.9)	Ref		89 (100.0)	Ref	
Yes	5 (2.1)	4 (2.1)	0.99	0.25-3.94	4 (4.1)	1.79	0.42-7.66	0 (0.0)	[d]	
Ever try for one year or more										
No	184 (76.3)	155 (79.5)	Ref		76 (78.4)	Ref		70 (78.7)	Ref	
Yes	57 (23.7)	40 (20.5)	0.85	0.53-1.37	21 (21.6)	1.02	0.53-1.95	19 (21.3)	0.86	0.45-1.64
Time to index pregnancy										
Not trying	119 (49.4)	94 (48.5)	0.75	0.48-1.20	50 (51.5)	0.88	0.48-1.62	40 (45.5)	0.71	0.37-1.33
Less than one year of trying	95 (39.4)	82 (42.3)	Ref		37 (38.1)	Ref		40 (45.5)	Ref	
One year or more of trying	27 (11.2)	18 (9.3)	0.77	0.39-1.53	10 (10.3)	1.21	0.48-3.03	8 (9.1)	0.67	0.27-1.70
LCA infertility[c] (w/mat age)										
No	222 (92.1)	177 (90.8)	Ref		88 (90.7)	Ref		80 (89.9)	Ref	
Yes	19 (7.9)	18 (9.2)	1.23	0.57-2.62	9 (9.3)	1.52	0.55-4.25	9 (10.1)	1.19	0.45-3.19
LCA infertility[c] (w/o mat age)										
No	235 (97.5)	185 (94.9)	Ref		92 (94.8)	Ref		84 (94.4)	Ref	
Yes	6 (2.5)	10 (5.1)	2.35	0.80-6.92	5 (5.2)	1.75	0.47-6.52	5 (5.6)	3.06	0.77-12.10

a Logistic regression models were adjusted for child's age, sex, and gestational age as well as maternal age, race, education, and household income
b Prior to or before knowledge or pregnancy
c Infertility predicted from the latent class model
d Sample size too small for logistic regression analysis

Table 4: Association between childhood GCT and infertility and infertility treatment (Males only)

	Controls n (%)	Cases n (%)	OR[a]	95% CI	Gonadal n (%)	OR[a]	95% CI	Non-gonadal[d] n (%)
Prior fetal loss								
None	146 (80.7)	70 (84.3)	Ref		39 (83.0)	Ref		28 (90.3)
One	26 (14.4)	11 (13.3)	0.71	0.30-1.70	7 (14.9)	1.06	0.37-3.09	3 (9.7)
Two or more	9 (5.0)	2 (2.4)	[d]		1 (2.1)	[d]		0 (0.0)
History of multiple birth								
None	174 (96.1)	82 (98.8)	Ref		46 (97.9)	Ref		31 (100.0)
At least one multiple birth	7 (3.9)	1 (1.2)	[d]		1 (2.1)	[d]		0 (0.0)
Maternal age								
< 27	68 (37.6)	36 (43.4)	1.37	0.70-2.67	20 (42.6)	1.55	0.66-3.68	13 (41.9)
27-34	99 (54.7)	35 (42.2)	Ref		20 (42.6)	Ref		15 (48.4)
≥35	14 (7.7)	12 (14.5)	2.88	1.13-7.37	7 (14.9)	3.70	1.12-12.24	3 (9.7)
Use of OS drugs[b]								
No	175 (96.7)	82 (98.8)	Ref		46 (97.9)	Ref		31 (100.0)
Yes	6 (3.3)	1 (1.2)	[d]		1 (2.1)	[d]		0 (0.0)
Ever try for one year or more								
No	149 (82.8)	66 (79.5)	Ref		36 (76.6)	Ref		26 (83.9)
Yes	31 (17.2)	17 (20.5)	1.30	0.61-2.75	11 (23.4)	1.80	0.70-4.64	5 (16.1)
Time to index pregnancy								
Not trying	71 (39.4)	40 (48.2)	1.17	0.64-2.14	18 (38.3)	0.88	0.41-1.90	18 (58.1)
Less than one year of trying	88 (48.9)	37 (44.6)	Ref		24 (51.1)	Ref		12 (38.7)
One year or more of trying	21 (11.7)	6 (7.2)	0.56	0.17-1.81	5 (10.6)	1.09	0.27-4.36	1 (3.2)
LCA infertility[c] (w/mat age)								
No	167 (92.3)	77 (92.8)	Ref		42 (89.4)	Ref		31 (100.0)
Yes	14 (7.7)	6 (7.2)	0.72	0.21-2.48	5 (10.6)	1.68	0.39-7.29	0 (0.0)
LCA infertility[c] (w/o mat age)								
No	172 (95.0)	82 (98.8)	Ref		46 (97.9)	Ref		31 (100.0)
Yes	9 (5.0)	1 (1.2)	[d]		1 (2.1)	[d]		0 (0.0)

a Logistic regression models were adjusted for child's age, sex, and gestational age as well as maternal age, race, education, and household income
b Prior to or before knowledge or pregnancy
c Infertility predicted from the latent class model
d Sample size too small for logistic regression analysis

Chapter 4

Background: DNA methylation after assisted reproductive technology

Several studies have found that children born after assisted reproductive technology (ART) are more prone to severe disorders caused by abnormal genomic imprinting[3-12]. Although imprinting disorders in children born after ART are still very rare in an absolute sense, the fact that these diseases are more common could indicate a more widespread disruption of epigenetic mechanisms. These disruptions could potentially manifest themselves as an increased propensity for childhood cancer as well as adult onset diseases such as cancer and heart disease that are thought to be epigenetically mediated[163, 164]. With the growing number of children being conceived after ART, over 50,000 children per year in the United States[1], more research is needed to assess potential health risks.

Epigenetics

Epigenetic traits are defined as mitotically or meiotically heritable phenotypes that do not alter the actual DNA sequence[165]. There are three currently identified types of epigenetic mechanisms; DNA methylation, histone modification and chromatin remodeling, and small, non-coding RNAs (ncRNA). These mechanisms work together to regulate gene expression patterns. DNA methylation as well as histone modification and chromatin remodeling influence the transcription while ncRNAs may affect transcription or interfere with translation[166]. This review focuses on DNA methylation rather than

other mechanisms of epigenetic regulation since the current literature on ART and epigenetics has only examined DNA methylation and this process is involved in the gene regions of interest.

DNA methylation

DNA methylation is a broad type of epigenetic modification in which the cytosine in CpG sites is methylated. Methylation involves the addition of a methyl group from S-adenosylmethionine to the 5-carbon position of a cytosine and is acquired and maintained through various DNA methyltransferases (DNMTs) and other signaling processes. Three functional DNMTs have been discovered in humans. DNMT3A and DNMT3B are thought to be involved primarily in *de novo* methylation whereas DNMT1 is primarily responsible for maintenance of methylation through its affinity for hemimethylated DNA[167]. In addition to DNMTs, histone modification and ncRNAs may also be involved in *de novo* methylation[167].

Most of the CpG dinucleotides within mammalian genomes are methylated[168]. However, methylation at these sites varies depending the specific function and location of the CpG site. For example, CpG islands located near gene promoters and housekeeping genes are generally unmethylated (no effect on expression), whereas promoters and regulatory regions of transposable elements CpG sites are usually methylated (silencing expression), and, in imprinting control regions (ICRs), the CpG sites are differentially methylated (silencing one allele)[169]. Regardless of location, however, CpG sites that are methylated can have an impact on cellular function including transcription inhibition and silencing of repeat elements[169]. Regulation of gene expression through DNA methylation

can occur through different mechanisms such as interference or recruitment of binding proteins related to transcriptional activation or repression[167].

Methylation of non-imprinted genes is reset after fertilization through active demethylation of the paternal genome and passive demethylation of the maternal genome[170]. Remethylation of the genome takes place in a tissue specific manner after implantation leading to differential expression of certain genes in various tissues and cells[170]. DNA methylation is transmitted mitotically to daughter cells in a highly persistent manner. In fact, twin studies have shown that locus specific methylation is heritable and is more concordant in monozygotic twins compared to dizygotic twins[171, 172]. Although normally maintained, this process is reversible since methylation abnormalities are seen in cancer cells and tends to decrease with age[173-175]. For example, a study examining methylation changes over time was performed in twins in which older monozygotic twins were less concordant for global measures of methylation compared to younger monozygotic twins[176].

Methylation is not a dichotomous trait, but rather is quantitative with different levels seen in both normal and syndromic children and adults[171, 177-179]. Thus, subtle difference in methylation may be important from a public health standpoint and could be indicative of future disease susceptibility[177, 180].

Genomic Imprinting

Imprinting is a specific type of epigenetic effect in which expression of one allele is blocked resulting in parent-of-origin specific expression. Most imprinted genes are found in clusters rather than as individual genes. Within clusters of imprinted genes,

specific regions regulate gene expression of the cluster either partially or completely. Areas that regulate several genes in imprinted clusters are referred to as ICRs[181]. In addition to ICR there are regions within imprinting clusters that are differentially methylated, which also include ICRs, these regions are called differential methylated regions (DMRs) and may influence only one gene within the cluster. For example, the ICR KvDMR1 is thought to regulate expression of several genes including KCNQ1, CDKN1C, ASCL2, PHLDA2, TSSC4, and SCL22A1L[182, 183]. In imprinted regions with several DMRs there are usually only one or two ICRs. Other DMRs may be present, but these control expression of a single gene and are reset during the second demethylation period of development. For example, in the IGF2/H19 region there are four DMRs that have been identified in the mouse. One study examined methylation in the maternal and paternal alleles for these DMRs throughout early development. The authors found that for the three paternally methylated genes IGF2 DMR1 and IGF2 DMR2 lost methylation during early development while H19 DMR maintained methylation throughout indicating that methylation at IGF2 DMR1 and DMR2 occurs somatically and methylation in at H19 DMR is set and maintained in the germline[184]. This study suggests that H19 DMR is the ICR for this region while IGF2 DMR1 and IGF2 DMR2 are DMRs but not ICRs.

The function of imprinting in mammals is not fully understood. However, imprinting may be a specific mechanism for the regulation of growth, since many imprinted genes relate to growth and development. Imprinted genes that are paternally expressed are generally growth promoting while imprinted genes that are maternally expressed are generally growth inhibitory[185].

Imprinting is also different from DNA methylation both in the establishment of the epigenetic marks and in its stability. DNA methylation is reset during two different periods. The first period is during development of the primordial germ cells. It is during this period that ICRs are thought to be labeled through DNA methylation and possibly other epigenetic marks[170]. In the male germ cells imprinting occurs at an early stage of development and is complete by the time round spermatids are formed[186]. In the female germ line, however, imprinting is not complete until the oocyte is fully mature, so the use of oocytes from hyperstimulation and the maturation of oocytes in vitro could have an impact on imprinting in the subsequent offspring[187]. Methylation is reset again around the time of implantation of the fertilized egg. However, imprinted genes are not thought to be subject to this second demethylation and remethylation[170]. Imprinting is also more stable than DNA methylation. For example, a study examining the IGF2/H19 imprinting locus observed little difference in methylation over time[171]. Even though imprinting is generally stable, loss of imprinting (LOI) does occur, most notably in diseases such as cancer. LOI may in fact be a risk factor for certain types of cancer including Wilms tumor and colorectal cancer[174]. It is also possible that imprinting is altered by maintenance defects resulting in a mosaic LOI[118]. Thus early embryo effects even after the time of the establishment of imprints could still lead to mosaic LOI.

Imprinting disorders and ART

LOI can lead to a variety of disorders depending on the particular gene, or set of genes involved. There are several different types of molecular defects that can lead to LOI including a mutation in an imprinted gene, uniparental disomy (UPD), or epigenetic

defects. Mounting evidence suggests an increase in rare imprinting disorders in children conceived through infertility treatment, particularly ART. Increases in Beckwith-Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS), Angelman syndrome (AS), and retinoblastoma have been observed in several studies[3-12, 16, 188, 189]. However, these studies have been relatively small given the rarity of these imprinting disorders and have suffered from various methodological limitations. Not all studies have consistently linked imprinting disorders and ART though. A study that used the entire birth cohort of Denmark from 1995 -2001 found no cases of imprinting disorders in those children born after IVF where one case was expected[24]. There is also some evidence that imprinting disorders are increased due to parental infertility rather than ART. One study examined infertility (time to pregnancy > 2 years) and infertility treatment combined and found an increased risk of BWS in children born to parents with infertility problems regardless of treatment[188].

Beckwith-Wiedemann syndrome

BWS is a growth disorder with symptoms including macrosomia, macroglossia, visceromegaly of abdominal organs, and hemihyperplasia. It is estimated to have a population incidence of about 1 in 13,700[190]. Children diagnosed with BWS are at increased risk for childhood cancers, particularly embryonal tumors, with Wilms tumor and hepatoblastoma being the most common diagnoses. Tumor risk in the first ten years of life for those with BWS is estimated to be between 4% and 21%[191-195]. The defect that causes BWS is variable, with about 20% caused by UPD, 10% from mutations in the

CDKN1C gene, 2-7% from H19 DMR gain of methylation, 50% from loss of methylation at KvDMR, and 13-15% due to unknown causes[190].

A combination of case series, BWS registry studies, and ART cohorts have all shown increases in BWS after ART[3, 4, 6-12]. Molecular analysis has indicated that children conceived through ART who developed BWS have an increased rate of hypomethylation of KvDMR[8, 10-14]. One study found a patient with both hypermethylation at H19DMR and hypomethylation at KvDMR[6]. Another study reported that 24 out of 25 children with BWS conceived through ART had loss of methylation at KvDMR[15]. These results suggest an overrepresentation of children with this particular defect since only about 50% of children with BWS are expected to show loss of methylation at KvDMR[196]. In children with BWS conceived through ART, it is estimated that over 95% have hypomethylation at KvDMR[190].

Angelman syndrome

AS has an estimated prevalence between 1/10,000 and 1/20,000[197]. Characteristics of this disease include severe developmental delay, speech impairment, ataxia, apparent happy demeanor, and epileptic seizures[198]. Various genetic and epigenetic mechanisms can result in AS with about 70-75% attributable to deletion in the 15q11-13 region, 2-3% to UPD, 3-5% to abnormal methylation patterns, 5-10% to a mutation in the UBE3A gene, and 12-15% with unknown mechanism[199]. Children with AS generally display a normal phenotype at birth, but symptoms may begin to become apparent during the first year of life[198].

Several studies have examined AS in children conceived through ART. Two studies found an increased incidence of fertility problems, infertility treatment, and ART in a cohort of AS children[7, 188]. Another study reported a case series of two children who developed AS after conception with ART[5]. Molecular analysis was performed in two studies; in one study 1 out of 3 patients had hypomethylation of SNRPN while the other study found both children with AS conceived through ART to have hypomethylation at the SNRPN locus[5, 10]. These results are surprising since in AS hypomethylation of SNRPN is rare accounting for less than 5% of all cases[5].

Silver-Russell syndrome

SRS presents with many characteristics that are clinically opposite of BWS, such as low birth weight and poor postnatal growth. Other common features of SRS include asymmetry, urogenital abnormalities, macrocephaly, and a small, triangular face[200]. Estimates of incidence vary widely from 1/3,000 to 1/100,000[201]. Molecular findings in SRS have been diverse with genetic and epigenetic mutations found on two separate chromosomes. Approximately 10% of SRS is thought to be due to UPD of chromosome 7 and between 35-65% of cases are due to aberrant methylation in the H19/IGF2 ICR while the molecular basis for others with SRS is unknown[200].

A few cases of SRS in children conceived through ART have been reported in the literature[16, 202-204]. Two studies have found differential methylation in a single child with SRS conceived through ART. One study found hypermethylation in the DMR of the PEG1/MEST region on 7q32.2[16]. The other study found hypomethylation of the H19 promoter in a child conceived through ART with SRS characteristics[17].

Retinoblastoma

Retinoblastoma is a cancer of the retina that usually develops during the first two years of life. Although not an imprinting disorder in the typical sense, retinoblastoma has been grouped (probably inappropriately) with imprinting disorders in the ART literature, but not elsewhere. While there is some evidence of differential expression between the maternal and paternal alleles, the RB1 gene is not generally monoallelically expressed since this would lead to increased susceptibility to cancer[205]. Most cases of retinoblastoma are due to mutations in the RB1 tumor suppressor gene, but there is also evidence of hypermethylation of the RB1 gene in retinoblastoma[206]. However, this hypermethylation is thought to be somatic rather than germline. A set of studies estimated that about 13% of sporadic unilateral tumors have hypermethylation in this gene[206]. Approximately 300 children and adolescents under the age of 20 years are diagnosed with retinoblastoma in the United States each year[61]. Few studies have examined retinoblastoma and those that have presented somewhat conflicting results. There have been a few case series that reported a potential link between retinoblastoma and infertility treatment[207-209]. A study from The Netherlands reported in 2003 and updated in 2009 found a potential increase in retinoblastoma after parental infertility treatment, but only for the earlier time period[189, 210]. Another study found no cases of retinoblastoma in 176 children conceived through IVF[211]. Retinoblastoma was also among the diseases examined in the Danish cohort with no cases reported in children conceived through IVF[24].

DNA methylation in non-syndromic children and ART

Few studies examined differential methylation in children who did not show signs of severe imprinting disorders and most are based on very small sample sizes. One study, which examined differences in methylation in the PWS/AS-IC region, found no differential methylation in a series of 92 ART children[212], but did not include any spontaneously conceived children for comparison. Two studies examined placentas from ART births, one assessed gene expression found some indication of down regulation of the H19 and CDKN1C genes in the ART group but no difference in expression of the IGF2 or KCNQ1OT1 genes[213]; the other study showed similar results using methylation analysis including down-regulation of H19 and CDKN1C, but no change in IGF2[214]. One study examined differential methylation in buccal cells of children conceived through ICSI and who were small for gestational age. Out of 19 children only one child was found to have hypermethylation at KCNQ1OT1 and PEG1 whereas no controls had a similar pattern[215]. Another study examined peripheral blood samples in 12 children conceived through ART and 22 children conceived spontaneously. They found three ART children and no non-ART children with hypomethylation at KvDMR[216]. A final study examined gene expression through a methylation bead-array platform; the GoldenGate Array by Illumina[217]. They examined 1536 CpG sites in over 700 genes using placenta and cord blood samples and found an overall lower level of methylation at CpG sites in the placenta samples and a higher level of methylation in cord blood of in vitro conceived children. Many genes were found to have differential methylation in at least on CpG site in both cord blood and placenta samples.

In addition, levels of circulating IGF2 have been found to be elevated in children born after ART compared to spontaneously conceived children (850.3 μg/liter vs. 772.8 μg/liter, respectively (p= 0.03)) [218]. The higher level of circulating IGF2 could be due to a number of different mechanisms, one of which is differential methylation patterns. However, it is not clear that the difference observed in serum IGF2 would be clinically important. This result has also not been examined in other studies, so it is possible that it is merely a chance finding and does not represent an actual difference in IGF2 levels.

Imprinting, methylation, and cancer

Since DNA methylation and genomic imprinting are factors that control gene expression and cell proliferation, it may be expected that abnormal methylation and LOI would be seen frequently in cancerous tissue samples. In fact, one of the most notable features of tumor cells is their overall hypomethylation that occurs in repetitive elements causing instability; predisposing DNA to further mutations and damage[18]. Another change in methylation during carcinogenesis is hypermethylation of particular genes that effectively silences gene transcription. This silencing occurs in DNA repair and cell-cycle regulation genes that cause abnormal growth and unchecked DNA damage[18]. LOI is another common defect in tumor cells since many imprinted genes are associated with growth and cell cycle regulation. For example LOI in the IGF2 gene in cancer cells is thought to further tumor formation by inhibiting apoptosis[164].

Epigenetic changes on a global scale or within particular genes could be an early sign of carcinogenesis and have been proposed as early diagnostic markers of disease[22, 23]. The IGF2 imprinting region, for example, is associated not only with BWS, but also

with many cancers including Wilms tumor, colorectal, bladder, and breast cancer[19, 20]. Interestingly, one study examining colorectal cancer risk found LOI in the IGF2 region to be much more common in peripheral blood lymphocytes in those with a family history of colorectal cancer compared to those with no such family history[177]. Thus, this marker could be a candidate for early signs of colorectal cancer and, potentially, other cancers as well.

Causes of differential methylation and imprinting after parental infertility or its treatment

Differences in DNA methylation patterns could be the results of several different aspects of infertility itself or of infertility treatment. Possible avenues of methylation differences include ovarian stimulation, IVF culture medium or other in vitro manipulation, use of ICSI with abnormally methylated sperm, or other factors relating to the underlying cause of infertility. There is evidence that each of these affect embryonic DNA methylation from mouse studies and, to a lesser extent, human studies.

Perhaps the most likely cause of methylation differences in children born after infertility treatment is ovulation stimulation. As mentioned previously, the timing of establishment of imprinting marks overlaps with the timing of ovarian stimulation treatment for infertility. Thus, interventions in oocyte maturation could have a profound impact on imprinting and methylation. Most of the literature on the effect of ovarian stimulation is limited to mice, however one study examined human oocytes and other human studies have implied that ovarian stimulation could be the cause of imprinting disorders. One study examining mouse blastocysts after ovarian hyperstimulation found differences in expression of H19[219]. Another study found differences in expression of

SNRPN and H19 in mouse placenta, but not in the embryos themselves[220]. A third study examining the effect of ovarian stimulation in mice and humans found a dose dependent effect of stimulation hormones on expression of SNRPN, PEG3, KCNQ1OT1, and H19 in blastocyst embryos[154]. In observational epidemiological studies, the contribution of ovulation stimulation to imprinting and methylation defects is difficult to disentangle since almost all ART procedures are performed after ovulation stimulation. However, in a study using the BWS registry in the U.S. the only common factor in 19 patients born after ART who developed BWS was that all mothers had ovulation stimulation and at least one partner had infertility (although the reasons for infertility were diverse) [4].

Another possible cause of epigenetic disruption is through the culture medium used in IVF procedures. Studies analyzing various media have been primarily performed in mice and have found different levels of expression of imprinted genes depending on the medium used[221, 222]. Although there has been no direct study of imprinting or methylation difference in humans, research has been done that has shown differential outcome (implantation rate, live birth rate, birth weight) depending on culture media[223-225]. In addition to the composition of the culture medium, the time in culture could adversely affect imprinting. One study indicated that prolonging culture time led to demethylation of the imprinted SNRPN gene in mouse embryos[226].

The use of sperm from infertile men through ICSI represents yet another possible mechanism of abnormal imprinting and methylation. It has been demonstrated that men with infertility have sperm with a higher rate of DNA damage and that this damage can have an impact on ART outcomes such as pregnancy loss[227]. Recent evidence suggests the possibility of methylation changes in ICRs in men with oligozoospermia[117, 228-230].

One study found that the same aberrant DNA methylation patterns found in the sperm was also present in the embryo suggesting the possibility for inheritance of epigenetic mutations[231]. Many of these studies found abnormal imprinting in loci associated with imprinting disorders such as IGF2, H19, and LIT1.

Finally, other studies not looking specifically at male factor infertility have found that epigenetic defects after ART could be due to infertility rather than ART treatment. A recent study in The Netherlands indicated the same relative risk estimates for imprinting disorders in children born after ART and for children born to couples who had at least one child born after a fertility problem[7]. A study in Germany also found that fertility problems alone could increase the risk of imprinting disorders in offspring[188].

Conclusion

Based on the studies performed so far, it appears that not only is there an increase in imprinting disorders, particularly BWS and AS, after ART, but also that the specific mutations involved in these disorders arise from abnormal DNA methylation at ICRs rather than from DNA mutations or UPD. Since imprinting disorders are rare conditions, a small increase in incidence would not be necessarily important or have an impact on the clinical treatment of infertility; however, the possibility of subtle epigenetic abnormalities that could enhance disease susceptibility is a greater concern because the effects could be more widespread. While there is a lot still unknown about the impact of imprinting and methylation in regards to future disease development it is imperative to study these processes to better understand their relationship to disease and to help quantify potential risks.

Chapter 5

Similar DNA methylation levels in specific imprinting control regions in children conceived with and without ART

Introduction: While a possible link between assisted reproductive technology (ART) and rare imprinting disorders has been found, it is not clear if this is indicative of subtler disruptions of epigenetic mechanisms.

Methods: Children conceived through ART and children conceived spontaneously were recruited for this study. Information about reproductive history, demographic factors, birth characteristics, and infertility treatment was obtained from maternal interview and medical records. Peripheral blood lymphocytes and buccal cell samples were collected from participating children. Methylation analysis was performed on five loci using pyrosequencing. Statistical analysis of methylation differences was performed using linear regression with generalized estimating equations. Correlation between lymphocytes and buccal cell samples was also assessed.

Results: Total sample size was 67 ART children and 31 non-ART children. The data were consistent, for lymphocyte samples each locus showed no statistical difference between groups. Possible differences were found in buccal cell samples for IGF2 DMR0 (Difference: 2.33; 95% CI: -0.08, 4.82; $p = 0.06$) and IGF2R (Difference: -2.83; 95% CI: -5.88, 0.22; $p = 0.07$). Subgroup analysis indicated potential lower methylation in those whose parents used ART for unexplained infertility. Correlation between lymphocyte and buccal cell samples was very low for all loci.

Conclusions: Observed differences in methylation between the ART and non-ART groups were very small in the two tissues examined. Little correlation was noted in methylation levels between lymphocytes and buccal cells. Differences in methylation could still exist in other tissues or at other loci.

Introduction

Use of assisted reproductive technology (ART), including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), is rapidly rising in the United States (US) and currently accounts for over one percent of all infants born each year[1]. A growing body of evidence suggests an increase in rare imprinting disorders such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), and Silver-Russell syndrome (SRS) in children conceived through infertility treatment, particularly ART. A combination of case series, BWS registry studies, and ART cohorts have all suggested increases in BWS after ART[3, 4, 6-12]. In addition to ART, parental infertility (time to pregnancy > 2 years) and infertility treatment have been suspected to increase the risk of BWS[188]. Other studies have suggested a potential increase in SRS and AS after parental infertility or infertility treatment[7, 188 5, 16, 202-204]. However, not all studies have consistently shown an increase for all imprinting disorders[3, 7, 10, 24].

In addition to an increase in incidence, studies indicate that the cause of the imprinting disorders in children born after ART is more likely to be due to an epigenetic event rather than a mutation or uniparental disomy (UPD). Molecular analysis has indicated that children conceived through ART who developed BWS have an increased rate of hypomethylation of KvDMR[6, 8, 10-15]. Specifically, it is estimated that over 95% of children with BWS conceived through ART have hypomethylation at KvDMR compared to the expected 50%[190, 196]. Children conceived through ART who develop SRS and AS are also noted to have rare methylation defects rather than mutations or UPD[5, 10, 16, 17].

Imprinting disorders are very rare and, even with a relative increase in incidence of these disorders, most children conceived through ART are healthy. However, a relative

increase suggests the possibility for more frequent, but subtler disruptions of epigenetic mechanisms. It has been suggested that such epigenetic disruptions could potentially manifest themselves as an increased propensity for childhood cancer as well as adult onset diseases such as cancer and heart disease that are thought to be epigenetically mediated[163, 164]. Some studies have suggested that there is differential methylation in various tissues in children born after ART compared to spontaneously conceived children in the 11q15.5 region[213-216]. Additional research to examine epigenetic disruptions in phenotypically normal children is needed to assess potential health risks.

We conducted the Imprinting and Methylation Patterns after ART (IMPART) study to examine DNA methylation in children conceived after ART treatment and compare methylation levels in this group to a group of children conceived spontaneously. The study focuses on differential methylation at the 11q15.5 region including loci at both H19/IGF2 and KvDMR and also includes a differentially methylated region (DMR) in the IGF2R gene located at 6q26. These loci are not only associated with BWS and SRS but also epigenetic disruptions in these regions have been implicated in many different types of cancer[232]. Thus, we were interested in pursuing this possible pathway to disease.

Materials and Methods

Study population

Two groups of children were recruited for this study; one conceived through ART (ART group) and the other born after spontaneous conception (SC group). ART children had to be conceived through IVF or IVF + ICSI with fresh non-donor oocytes. SC children must have been conceived without the use of any fertility drugs or treatments. In

the case of multiple births in either the ART or SC group only one child was selected for participation in the study. Children diagnosed with BWS, AS, Prader-Willi syndrome, or retinoblastoma were excluded from the study.

ART children and their mothers were recruited from the University of Minnesota Reproductive Medicine Center (UMRMC). Mothers who conceived through ART and reported a live birth between March 2005 and December 2008 were contacted about participation of their child through a letter and follow-up phone call. Out of 328 women identified as potentially eligible through the UMRMC, 99 agreed to have their child participate and 53 children completed the clinic visit.

SC children were recruited through advertisements posted in and around the University of Minnesota. Similar to the ART children, SC children had to have been born between March 2005 and December 2008. A total of 45 women agreed have their child participate in the study and 31 children completed the clinic visit prior to the close of the study. This study was approved by the institutional review boards at the University of Minnesota. All mothers provided informed consent for their child prior to participating in the study. (See Appendix D for additional recruitment details and Appendix E for initial letters and consent forms).

Sample Collection

Peripheral blood lymphocytes and buccal cell samples were collected either at a research clinic or at the participant's local clinic. Samples from the research clinic were delivered for processing immediately, while other clinic samples were sent to the lab via FedEx and were processed immediately upon receipt (usually within 24 hours of sample

collection). Up to 6 ml of blood was collected from each child through venipuncture in a tube containing acid citrate dextrose. Buccal cells were collected using Catch-All™ Sample Collection Swabs (Epicentre, Madison, WI, USA). Two swabs were collected from each child.

Data Collection

Information about ART and SC mothers and children was collected through a brief questionnaire as well as from delivery records (see Appendix E for the study questionnaires). In addition, for ART mothers, information on specific procedures was obtained from UMRMC records. The questionnaire collected demographic information, as well as maternal reproductive history and the participating child's birth characteristics. SC mothers were also asked about time to pregnancy to assess possible infertility that resolved without treatment. Delivery records were used to verify information on birth weight, gestational age, and maternal reproductive history. Information obtained from UMRMC records included indication for infertility, number of cycles of treatment, and specific procedure information.

Genetic regions analyzed

In this study, three regions of interest were examined for differential methylation: IGF2/H19, KvDMR, and IGF2R. These regions were selected based on their association with BWS and cancer. Specifically, we analyzed IGF DMR0 (3 CpGs), 3rd (11 CpGs) and 6th (16 CpGs) CTCF-binding site of H19 DMR, IGF2R (15 CpGs), and KvDMR (7

CpGs). The 5th CpG cite in the 6th CTCF-binding site of H19 DMR is known to be polymorphic and so was excluded from the analysis[230].

DNA extraction

DNA was extracted from frozen lymphocytes using the QIAamp DNA Mini kit spin protocol for blood and body fluids (Qiagen, Germantown/Gaithersburg, USA). DNA was extracted from buccal swabs using the Puregene DNA isolation for buccal brushes protocol (Qiagen, Germantown/Gaithersburg, USA).

Methylation analysis

Methylation analysis was performed using pyrosequencing techniques. The process was the same for all sites analyzed. Genomic DNA was isolated from the lymphocyte samples and treated with sodium bisulfite using the EZ methylation kit (Zymo research). This treatment actively changes unmethylated cytosines to uracils while leaving methylated cytosines unchanged. Primers and procedures for IGF DMR0, 3rd and 6th CTCF-binding site of H19 DMR, IGF2R were the same as in Boissonnas, et al[230]. Briefly, 1μL bisulfite treated DNA was combined with 1x HotStar Taq buffer that was supplemented with 1.6μM MgCl2, 100 μM dNTPs, 2 U HotStar Taq polymerase and 5 pmol of forward and reverse primers with one of the primers biotinylated. Denaturing was performed at 95°C for 15 minutes followed by 50 cycles of 30 s at 95°C. Annealing was performed at specific temperatures for each locus for 30 s followed by 20 s at 72°C. The extension step was performed for 5 min at 72°C. The protocol for the KvDMR region was different and based on Bourque, et al[233]. After bisulfite conversion, PCR was

performed using 1x HotStar Taq buffer, 0.2 mM dNTP, 5 pmol of each primer, 1.0 U HotStar Taq polymerase, and 2uL bisulfite converted DNA. Denaturing was performed at 95°C for 10 minutes followed by 40 cycles of 40 s at 95°C. Annealing was performed at 55°C for 40 s and 40 s at 72°C. The extension step was performed for 7 min at 72°C. After PCR amplification, pyrosequencing was performed for all regions using the PyroMark MD system, and analyzed using the accompanying software (Qiagen, Germantown/Gaithersburg, USA).

Methylation was assessed using the pyrogram generated by the pyrosequencing software. The percent methylation for each CpG was calculated by taking the peak height of the methylated cytosines divided by the sum of the peak height of the methylated and unmethylated cytosine. Several quality assurance tests were performed to assess the sequence generated by the pyrosequencing reaction against the expected sequence. Any suspected problem was analyzed and either a decision was made to accept the data or the entire sample was retested. CpG sites that consistently failed quality assurance tests were excluded from the analysis. We excluded one CpG site from H19 CTCF3, one CpG site from H19 CTCF6 and six CpG sites from IGF2R due to quality and polymorphism issues. Two children were excluded in locus-specific analysis due to unexpected sequences, one in the KvDMR locus and the other in the IGF2 DMR0 locus. (See Appendix F for additional sample processing information).

Data analysis

Descriptive data were compared between the two groups using chi-squared tests (or Fisher's exact tests) for categorical variables, and t-tests for continuous variables.

Differences in methylation between groups were analyzed using linear models with Generalized Estimating Equations (GEE). The GEE model accounts for the correlation between CpG cites within an individual. Each locus was considered separately. Multivariate models were constructed using variables possibly related to use of ART: maternal age, maternal education, household income and child's birth weight; and variables possibly associated with methylation: child's age and gender. All models also included a variable indicating day of the pyrosequencing run to control for any batch effects. Subgroup analysis was performed within the ART group to examine potential differences in methylation by type of infertility. Graphs were created using average methylation at each CpG site, adjusting for batch effects. Results are reported as group means, differences between groups, and 95% confidence intervals (CI) for the difference between groups. Correlation between lymphocyte and buccal cell samples was also assessed using Pearson's correlation coefficient for each CpG site at each locus. Sensitivity analysis was performed excluding the samples that were rerun after assay failure. All analysis was performed using SAS 9.1 (SAS Institute, Inc, Cary, NC).

Results

A total of 67 children were included in the ART group and 31 in the SC group. Of these, 53 ART children and 27 SC children provided a blood sample while 67 ART children and 30 SC children provided a buccal cell sample. Demographic factors of the two groups are presented in Table 1. Many factors were different between the two groups, with the ART group tending to have higher household income, increased

maternal age, and greater frequency of multiple birth. Children in the ART group tended to be younger and have lower birth weights.

Table 2 presents data on infertility diagnosis and treatment for couples in the ART group. Infertility diagnoses were fairly equally divided between female factor only (34%), male factor only (28%), and both male and female factors (26%). No medical explanation for infertility was found in 10% of couples. Most couples used ICSI procedures for at least some of the embryos (84%) and over 70% had two embryos transferred.

Methylation analysis

No large differences in methylation were found between the ART and SC groups in either lymphocyte or buccal cell samples (Figures 1 and 2). Table 3 displays the differences and 95% CI for the multivariate models. A marginally significant difference was seen in buccal cells only at IGF2 DMR0, with the ART group having higher methylation levels compared to the SC group (Difference: 2.33; 95% CI: -0.08, 4.82; p = 0.06). Larger differences were also observed for the IGF2R region; for buccal cells, this difference was marginally significant, showing a lower level of methylation in the ART group (Difference: -2.83; 95% CI: -5.88, 0.22; p = 0.07). However, due to the increased variability in methylation at this locus, the difference between groups was not statistically significant for lymphocyte samples. Estimates were similar when limited to those samples that were not rerun due to assay failure (see Appendix G).

In the subgroup analysis, couples with unexplained infertility had children that tended to have lower methylation levels compared with couple in which both partners

had an identified cause of infertility (see Table 4). Sample size was small, however, and no overall group comparison was statistically significant.

In all loci, correlation between methylation in lymphocyte samples and buccal cell samples was low (see Table 5).

Discussion

Overall, there was little difference in methylation between children conceived through ART and children conceived spontaneously. In subgroup analysis, couples with unexplained infertility tended to have lower methylation levels compared to couples in which both partners had an identified cause of infertility. While no studies, to the best of our knowledge, have examined differences in methylation by infertility diagnosis, several studies have examined male factor infertility and the potential to pass on epigenetic mutations at imprinted sites[231, 234, 235].

We found that methylation levels did not correlate well between lymphocyte and buccal sample at any of the examined loci. Few studies have examined differential methylation by tissue type. One study, however, found high correlation overall in methylation levels between tissue types, but also some specific genes displaying markedly different methylation levels within different tissues[236]. The reason for the low correlation in our study could be due to a limited range of values in the lymphocyte samples combined with much larger variability in buccal cell samples. With their lower variability, lymphocyte samples may be preferred in future studies exploring possible subtle differences in DNA methylation.

The observed differences in methylation between the ART and SC group were very small, indicating that our failure to detect differences was not due to a lack of power. For lymphocyte samples, average differences were around 1% or less between groups. Based on the variability of the samples, our analysis suggested that differences greater than 2-4 percentage points for all loci except IGF2R are unlikely. Methylation in buccal cell samples was more variable, with an average difference of around 2%. For these samples, differences greater than 5-7 percentage points appeared unlikely.

Other studies have examined differential methylation in various tissues of children born after ART with normal phenotypes. A few studies have indicated a normal methylation pattern at the imprinting control region for AS and Prader-Willi syndrome[212-214]. Studies have also examined the region 11q5.15 associated with BWS and found possible methylation differences within this region [213-216]. A final study used a methylation bead-array platform and examined 1536 CpG sites in over 700 genes using placenta and cord blood samples and found an overall lower level of methylation at CpG sites in the placenta samples of in vitro conceived children and a higher level of methylation in cord blood of in vitro conceived children[217]. Additional detail about these studies is presented in Table 6.

The current study was one of the first to examine methylation differences in buccal cells and peripheral blood lymphocytes in non-syndromic children conceived through ART and children conceived spontaneously. It also included two tissue types to examine differences and possible correlation in methylation levels. While sample size was not large, it was unlikely that we missed large differences in methylation based on our confidence intervals.

There are several caveats that should be addressed in this study. First, methylation abnormalities might be tissue specific rather than a global phenomenon. While it would be interesting to examine other tissues, it would not be ethical to obtain other tissue types in otherwise healthy children to test for tissue specific methylation patterns, since most collection procedures would be invasive. Unexposed individuals were a convenience sample rather than a random sample from a particular population. Women who use ART are more likely to be white, have higher incomes, and be better educated compared to infertile women who choose other or no treatment[237]. Although we observed differences between the ART and SC groups in our sample, the mothers in the SC group were more likely to be white and have higher income and education levels compared to the US population and thus may represent a good comparison group. In addition, only a limited number of genetic loci were evaluated in this study. Since this study was among the first of its kind, only those with the most *a priori* likelihood of an association were chosen to be examined to lower costs and maximize the potential for finding important associations.

Overall, only very small differences were observed in methylation level at all loci between groups. Some marginally significant differences in buccal cells may warrant additional follow up in other studies. Differences in methylation between children conceived through ART and those conceived spontaneously could still exist in other tissue types or at other loci. In examining methylation difference in the ART subgroup by infertility diagnosis, there was some indication of lower methylation levels in children born to parents with unexplained infertility. While no differences were found in this

study, additional studies will be important to assess possible changes in methylation patterns at other loci and with other tissues.

Table 1: Descriptive statistics by study group

Variable	ART group n = 67	SC group n = 31*	P-value Chi-square or t-test
Maternal Characteristics			
Race			
White	64 (95.5)	27 (90.0)	0.29
Non-white	3 (4.5)	3 (10.0)	
Education			
< College degree	10 (14.9)	10 (33.3)	0.09
College degree	26 (38.8)	11 (36.7)	
Advanced education	31 (46.3)	9 (30.0)	
Household income			
<40K	4 (6.0)	7 (23.3)	0.02
40K-<80K	18 (26.9)	10 (33.3)	
80K +	45 (67.2)	13 (43.3)	
Age at child's birth			
mean (SD)	34.1 (3.9)	29.6 (4.3)	<0.001
Time to pregnancy			
Not trying		9 (31.0)	
< 12 months		18 (62.1)	
≥ 12 months		2 (6.9)	
missing		1	
Child Characteristics			
Gender			
Female	32 (47.8)	13 (43.3)	0.69
Male	35 (52.2)	17 (56.7)	
Plurality			
Singleton	44 (65.7)	30 (100.0)	<0.001**
Twins+	23 (34.3)	0 (0.0)	
Year of birth			
2005	7 (10.4)	9 (30.0)	0.09
2006	23 (34.3)	8 (26.7)	
2007	23 (34.3)	10 (33.3)	
2008	14 (20.9)	3 (10.0)	
Birth weight			
mean (SD)	2996.1 (810.0)	3519.4 (686.2)	0.004
Age (years)			
mean (SD)	2.5 (0.97)	3.0 (1.00)	0.02

*One mother did not fill out the questionnaire

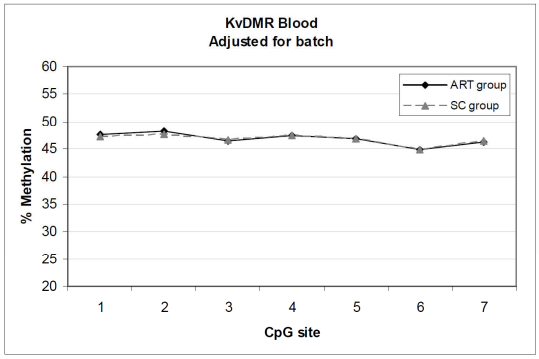
**Using Fisher's exact test

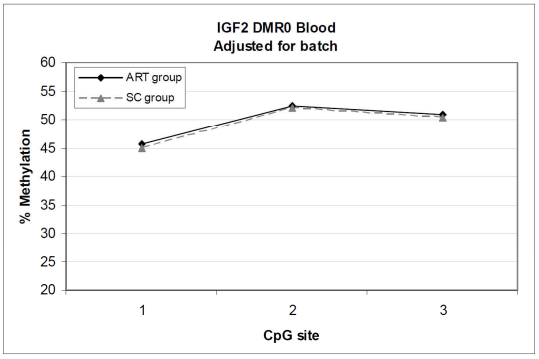
Table 2: Descriptive statistics for participants undergoing ART

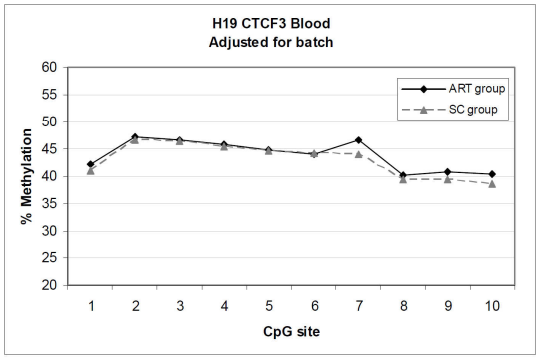
Variable	n = 61* n (%)
Type of infertility	
Female factors	21 (34.4)
Male factors	17 (27.9)
Both male and female factors	16 (26.2)
Unexplained	6 (9.8)
None	1 (1.6)
Specific female factors (n = 37)	
Uterine factor	6 (16.2)
Ovulation dysfunction	9 (24.3)
Tubal factor	7 (18.9)
Endometriosis	7 (18.9)
Diminished ovarian reserve	2 (5.4)
Multiple factors	6 (16.2)
Use of ICSI	
Yes	51 (83.6)
No	10 (16.4)
Number of embryos transferred	
1	1 (1.6)
2	43 (70.5)
3	16 (26.2)
4	1 (1.6)
Day of Transfer	
3	60 (98.4)
5	1 (1.6)
Length of infertility (months) mean (SD)	32.85 (20.81)

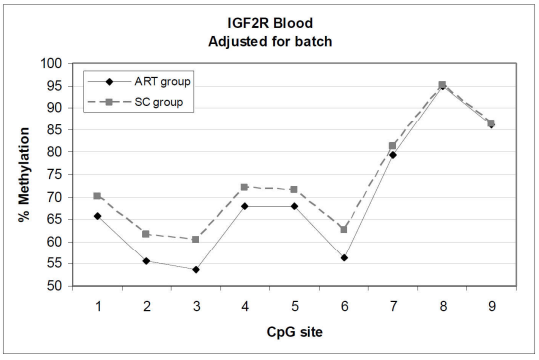
* Medical records for six subjects could not be accessed

Figure 1: Lymphocyte methylation by CpG site adjusted for batch effect









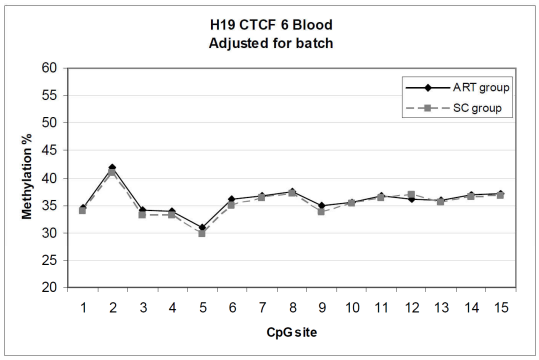
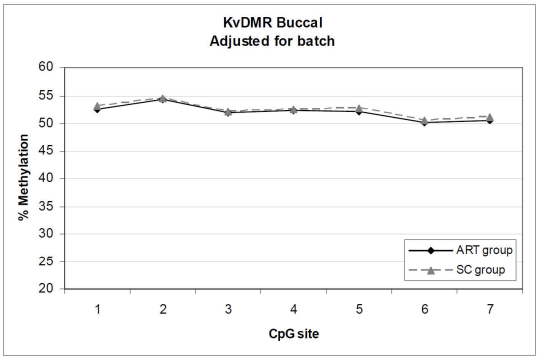
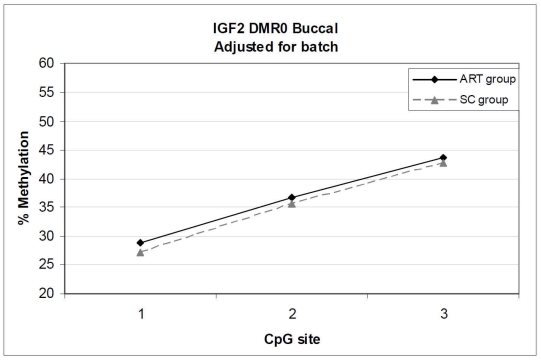
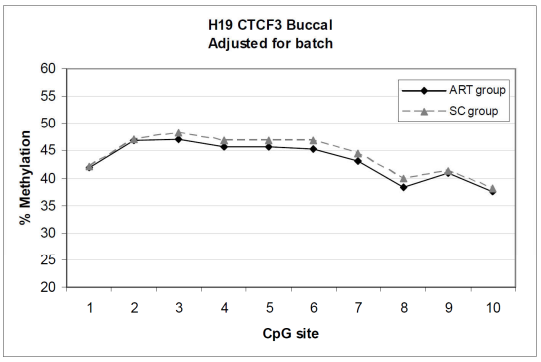
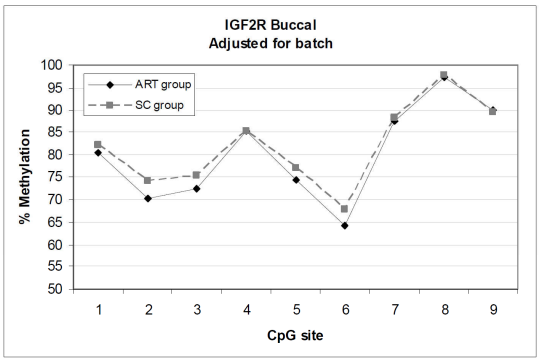


Figure 2: Buccal cell methylation by CpG site adjusted for batch effect









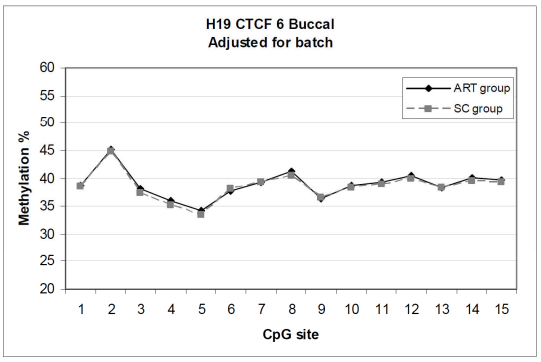


Table 3: Multivariate regression model results

Gene region of interest	ART group Mean*	SC group Mean*	Difference Estimate**	95% CI	p-value
Lymphocyte samples					
KvDMR	47.14	46.17	0.96	(-0.81, 2.74)	0.29
H19 CTCF3	43.15	42.25	0.90	(-0.70, 2.50)	0.27
H19 CTCF6	36.14	35.05	1.09	(-1.72, 3.90)	0.45
IGF2DMR0	48.82	48.93	-0.11	(-2.08, 1.85)	0.91
IGF2R	68.73	73.05	-4.32	(-9.52, 0.88)	0.10
Buccal samples					
KvDMR	51.54	49.66	1.89	(-2.69, 6.46)	0.42
H19 CTCF3	42.89	42.69	0.20	(-2.60, 3.00)	0.89
H19 CTCF6	38.42	36.37	2.05	(-2.34, 6.44)	0.36
IGF2DMR0	36.70	34.37	2.33	(-0.09, 4.75)	0.06
IGF2R	78.89	81.72	-2.83	(-5.88, 0.22)	0.07

*The reported mean is averaged over all CpG sites in the region of interest

**Adjusted for child's age, child's birth weight, child's gender, maternal age, maternal education, and household income

Table 4: Multivariate regression model results by infertility diagnosis

Gene region of interest	Diff (95% CI)* Female vs. Both**	Diff (95% CI)* Male vs. Both**	Diff (95% CI)* Unexp vs. Both**	Group comparison p-value
Lymphocyte samples				
KvDMR	0.03 (-1.75, 1.81)	-0.21 (-2.84, 2.43)	-2.71 (-6.40, 0.98)	0.51
H19 CTCF3	-0.96 (-2.96, 1.05)	-1.10 (-3.26, 1.06)	-2.62 (-5.99, 0.76)	0.56
H19 CTCF6	-0.89 (-3.82, 2.04)	1.01 (-2.68, 4.71)	-1.98 (-8.61, 4.66)	0.72
IGF2DMR0	-1.26 (-3.24, 0.71)	-1.03 (-3.59, 1.53)	-3.45 (-6.87, -0.04)	0.40
IGF2R	0.43 (-4.02, 4.88)	-3.40 (-7.48, 0.67)	-5.92 (-13.06, 1.22)	0.39
Buccal samples				
KvDMR	-5.50 (-10.86, -0.15)	-4.03 (-9.42, 1.37)	-7.40 (-14.66, -0.15)	0.14
H19 CTCF3	-1.63 (-5.29, 2.02)	-1.84 (-5.95, 2.27)	-4.72 (-9.41, -0.03)	0.37
H19 CTCF6	-3.52 (-8.37, 1.33)	-0.77 (-7.57, 6.02)	-5.16 (-12.33, 2.00)	0.30
IGF2DMR0	0.17 (-2.86, 3.19)	0.57 (-2.64, 3.78)	-1.34 (-5.93, 3.26)	0.82
IGF2R	0.76 (-1.80, 3.32)	1.31 (-0.94, 3.55)	-3.64 (-10.24, 2.95)	0.54

*Adjusted for child's age, child's birth weight, child's gender, maternal age, maternal education, and household income
** Infertility diagnoses were divided into the following groups: Female factors (Female), Male factors (Male), Both male and female factors (Both), and Unexplained (Unexp)

Table 5: Correlation between lymphocyte and buccal cell samples

Gene region of interest	n	CpG sites (n)	Min Corr	Median Corr	Max Corr
KvDMR					
Combined	76	7	-0.06	-0.02	0.03
ART group	50	7	-0.07	-0.03	0.03
SC group	26	7	-0.11	-0.02	0.07
H19 CTCF3					
Combined	77	10	-0.02	0.09	0.24
ART group	51	10	-0.08	0.14	0.25
SC group	26	10	-0.16	0.17	0.25
H19 CTCF6					
Combined	75	15	-0.08	0.01	0.07
ART group	50	15	-0.05	0.08	0.14
SC group	25	15	-0.20	-0.09	0.02
IGF2DMR0					
Combined	75	3	0.22	0.23	0.23
ART group	51	3	0.12	0.18	0.20
SC group	24	3	0.28	0.36	0.42
IGF2R*					
Combined	71	9	0.06	0.24	0.38
ART group	48	9	-0.01	0.17	0.34
SC group	23	9	0.05	0.21	0.59

* Excluded five influential observations (3 SC and 2 ART)

Table 6: Studies examining DNA methylation in non-syndromic children conceived through ART

Author	Year	Type of tx	Tissue type	Exposed	Unexposed	Gene/Region	Findings
Manning, et al	2000	IVF + ICSI	Blood	92	Reference sample from normal control	15q11-q13	Products from maternal and paternal alleles as expected
Neri, et al*	2008	ICSI singletons	Blood, placenta	53 blood 24 placenta	0 blood 20 placenta	15q11-13 blood 11p15.5 placenta	15q11-13: no difference from expected 11p15.5: down regulation of H19 and CDKN1c, no difference in IGF2 or KCNQ1OT1
Palermo, et al*	2008	ICSI	Blood, placenta	55 blood 56 placenta (37 IVF, 19 ICSI)	0 blood 37 placenta	15q11-13 blood 11p15.5 placenta	15q11-13: no difference from expected 11p15.5: down regulation of H19 and CDKN1c, no difference in IGF2 or KCNQ1OT1
Kanber, et al	2009	SGA ICSI	Buccal smears	19	29	KCNQ10T1 and IGF2/H19 (11p15.5), PEG1 (7q23), PEG3 (19q13.4), PLAGL1 (6q24), GTL2 (14q32.3),	1 SGA ICSI child with hypermethylation of KCNQ1OT1 and PEG1
Gomes, et al	2009	IVF or IVF+ICSI	Blood (PB), Cord blood/ placenta (CBP)	Total: 18 PB: 12 CBP: 6	Total 30 PB: 22 CBP: 8	KvDMR1 (11q15.5)	3 ART children with hypomethylation in PB, Lower meth in PB in ART, but not stat sig
Katari, et al	2009	IVF	Cord blood, Placenta	10	13	Genome wide 736 genes	Differentially methylated CpG sites tended to have higher methylation in cord blood and lower methylation in placenta

* Overlapping studies at the same institution

Conclusion

The null results presented here only apply to the narrow context of the studies conducted. Specifically, we found no relationship between parental infertility or its treatment and infant leukemia or pediatric germ cell tumors. Other childhood cancers may be associated with infertility. Similarly, while we found no differential methylation between the assisted reproductive technology (ART) and spontaneous conception (SC) groups at the loci examined in the final study, these findings due not exclude the possibility of differences at other sites or within other tissues.

These studies represent a relatively comprehensive examination of childhood cancer in children conceived through infertility treatment using both traditional and molecular epidemiological methods. Within this area of research it will be important to combine these types of studies in order to sort out potential associations and pathways to disease.

There were several positive attributes of each study. First, all studies were relatively large given the rarity of childhood cancer and ART. In both case-control studies, the sample sizes enabled results to be examined by potentially important etiologic subgroups. In the final study we were able to rule out large differences in methylation between our two groups. The three studies also benefited from the use of advanced epidemiological methods. In the case-control studies, use of latent class analysis provided a way to combine data over several potential measures of infertility or its treatment. Since many questions asked in case-control studies regarding infertility may be prone to error, the combination of several variables might mitigate

misclassification and provide a better assessment of infertility. In the final study, the use of generalized estimating equations made better use of the methylation data at each CpG site within a region of interest and provided more accurate estimates of error.

There are also several limitations to these studies that have been discussed in more detail within each study individually. The case-control studies are subject to recall bias and all three studies are prone to selection bias. Evidence of selection bias was seen in all studies though this bias was somewhat mitigated by the use of variables related to selection in the regression models. We also examined multiple exposures in the case-control studies and multiple outcomes in the methylation study. The use of multiple tests could lead to spurious findings. However, all studies were considered exploratory, so the few significant findings would need confirmation from other studies.

Additional studies exploring the relationship between childhood cancer, epigenetics and parental infertility are needed to further assess possible associations. Cancer-specific subtype studies would be helpful to identify potential association within rare cancer types. Follow-up to these studies could include molecular analysis of the particular cancer, to determine if the epigenetic profiles of tumors are different in children conceived with and without ART. If differences in the tumor profile are evident than this would provide evidence of different etiology in children conceived through ART.

Other molecular studies are also needed to examine more frequent, subtle epigenetic disruptions. Current and past studies have suffered from relatively low sample size and analysis of limited genetic regions. One promising approach is the use of

microarray technology to survey a wide variety of differentially methylated genes. One study found many genes with differential methylation using this approach, but only included 23 children total (10 ART and 13 non-ART)[217]. A follow up study with additional individuals using the same global platform could potentially confirm the differences seen in this study.

Another potential set of studies would be to examine methylation in different tissues. While some studies have included placenta and cord blood samples, few have used peripheral blood or buccal cell samples. Although buccal cells provide a convenient source of DNA, in our study, we found that there was greater variability in buccal cell samples and that they may not produce results similar to lymphocyte samples. Additional study could further examine this difference to determine the best tissue to examine.

The three studies presented here add to the literature by providing evidence of a null association between infant leukemia, pediatric germ cell tumors, and methylation at specific loci and parental infertility or infertility treatment. Although we found little evidence of a relationship between parental infertility and childhood cancer, there are still many interesting and productive avenues to explore in this area before a definitive determination of risk can be made.

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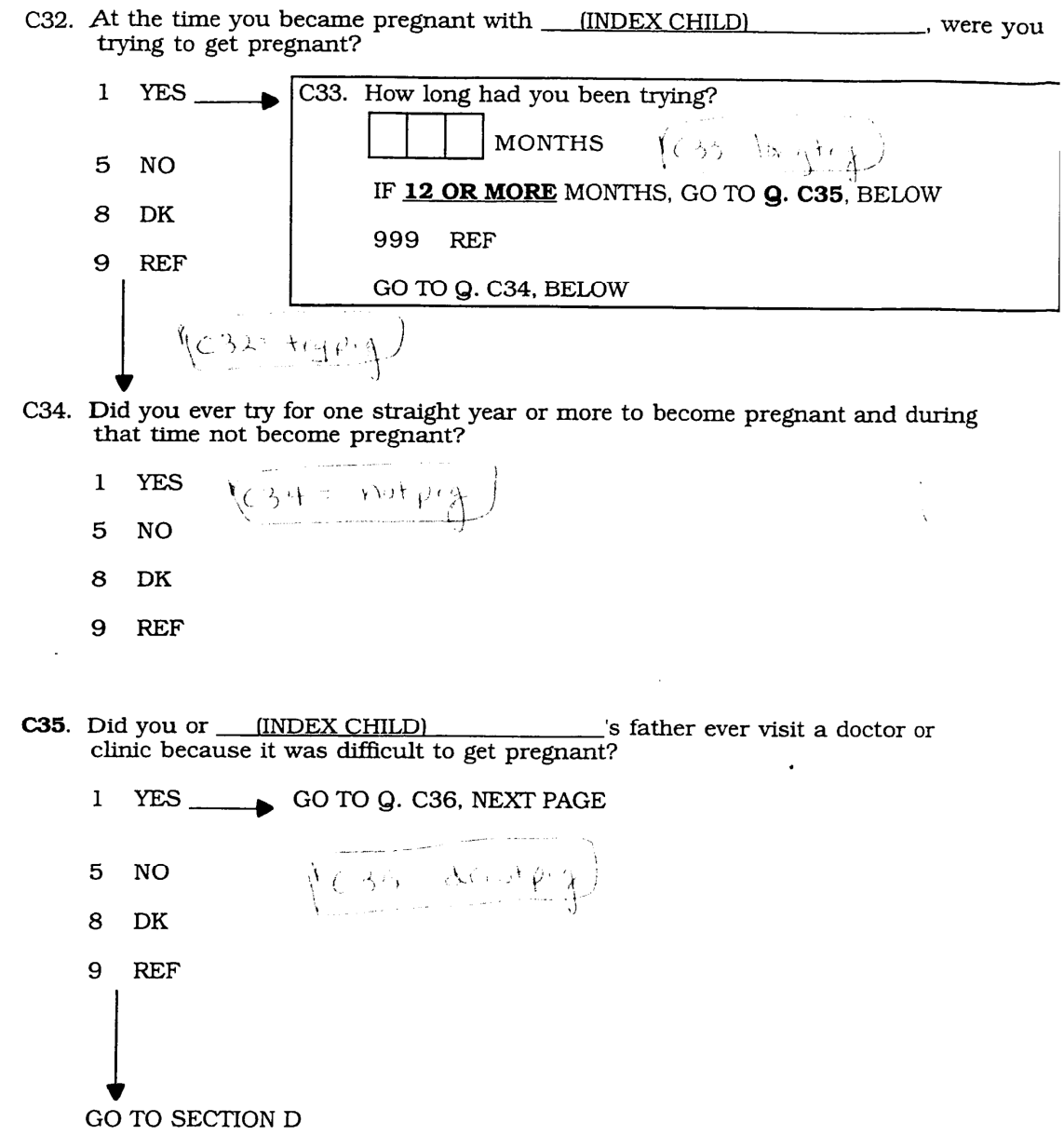
Appendix A: Infant leukemia and GCT infertility questions

Figure 1: Infant leukemia infertility questions



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C36. Did you have a surgical procedure to help you get pregnant?

1 YES
5 NO
8 DK
9 REF

C37. Did you receive tablets or injections to help you get pregnant?

1 YES → C38. What was that?

_____ A. ☐☐

_____ B. ☐☐

GO TO Q. C39, BELOW

5 NO
8 DK
9 REF

C39. Did you have some other treatment to help you get pregnant?

1 YES → C40. What was that?

_____ A. ☐☐

_____ B. ☐☐

GO TO Q. C41, NEXT PAGE

5 NO
8 DK
9 REF

GO TO Q. C41, NEXT PAGE

C41. Did the doctor tell you what the reason was for your difficulty in becoming pregnant?

1 YES → C42. Was the difficulty identified as a problem with your uterus or tubes, with your ovaries, with your hormones, was it a problem of the child's father, or was it something else?

C42 = reasonp

GO TO Q. C43, BELOW

5 NO

8 DK

9 REF

C43. Once you were pregnant with (INDEX CHILD), did you receive any medications or injections to help you stay pregnant?

C43 = stay preg

1 YES → C44. What was that?

C44 = meds preg

A.

B.

GO TO SECTION D

5 NO

8 DK

9 REF

GO TO SECTION D

Figure 2: Infant leukemia female hormones questions

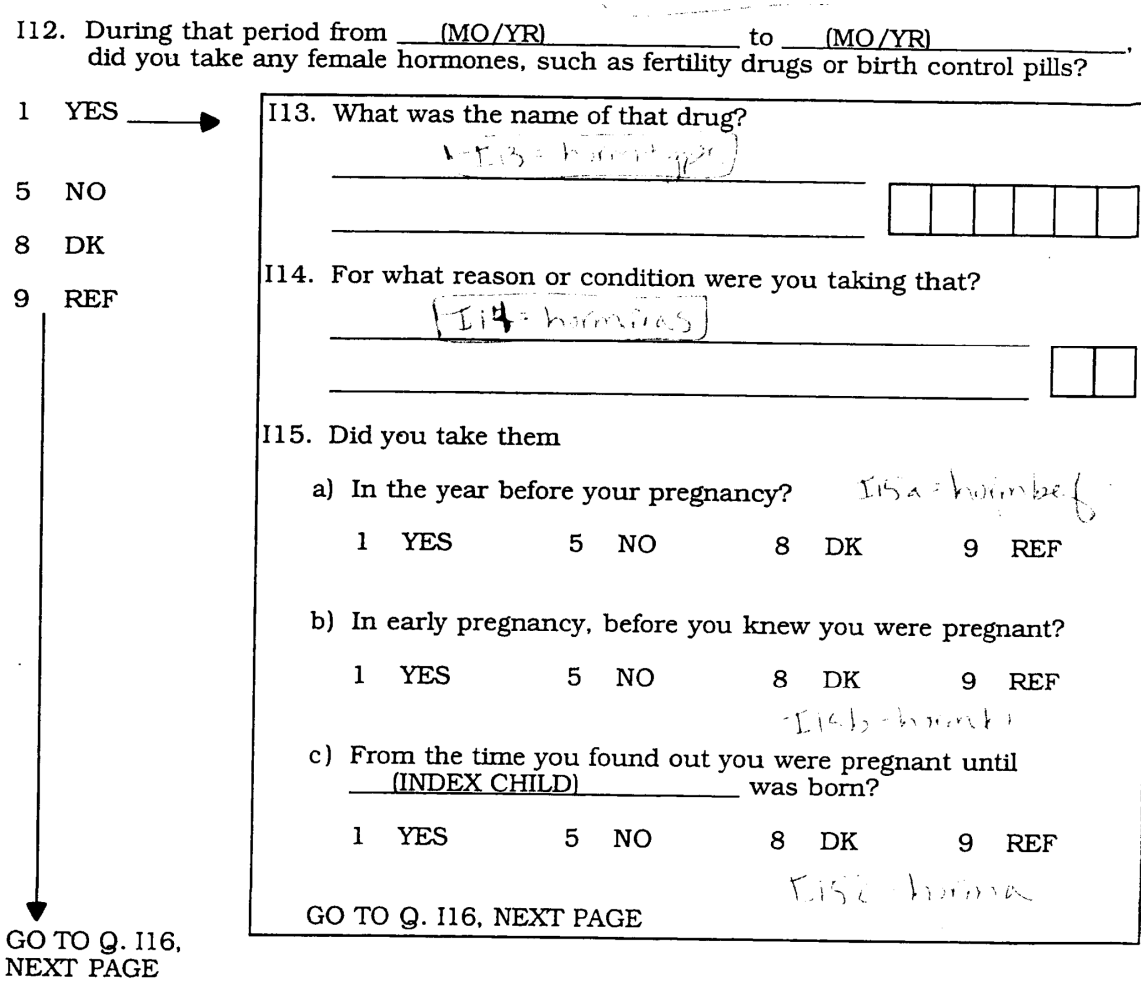

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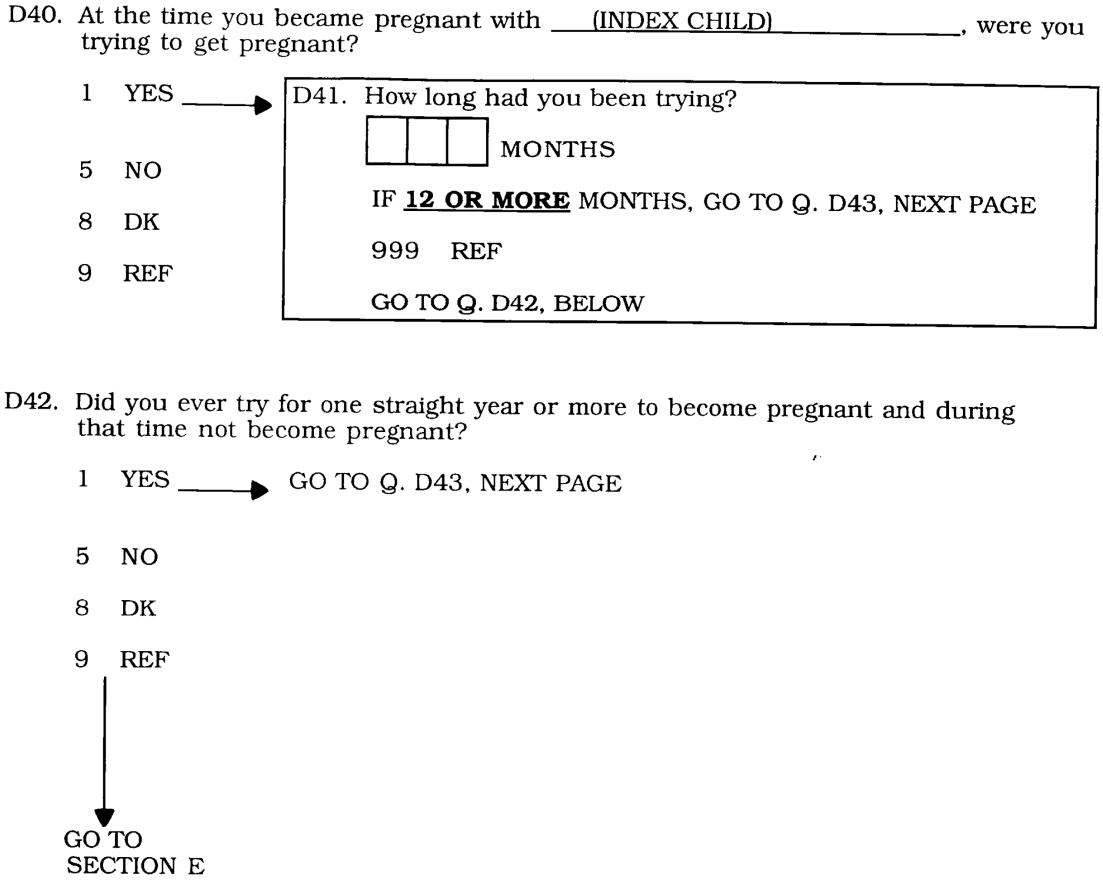
Figure 3: GCT infertility questions



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D43. Did you and (INDEX CHILD)'s father ever visit a doctor or clinic because it was difficult to get pregnant?

1 YES

5 NO

8 DK

9 REF

D44. Did you have a surgical procedure to treat the problem?

1 YES

5 NO

8 DK

9 REF

D45. Did you receive tablets or injections for the problem, such as Clomid?

1 YES

5 NO

8 DK

9 REF

D46. Did you have some other treatment for the problem?

1 YES → D47. What was that?

_____

_____ A. ☐☐

_____

_____ B. ☐☐

GO TO Q. D48, NEXT PAGE

5 NO

8 DK

9 REF

GO TO Q. D48, NEXT PAGE

D48. Did the doctor tell you what the reason was for your difficulty in becoming pregnant?

1 YES → D49. Was the difficulty identified as a problem with your uterus or tubes, with your ovaries, with your hormones, was it a problem of the child's father, or was it something else?

_____

_____

_____

_____

_____

_____

GO TO SECTION E

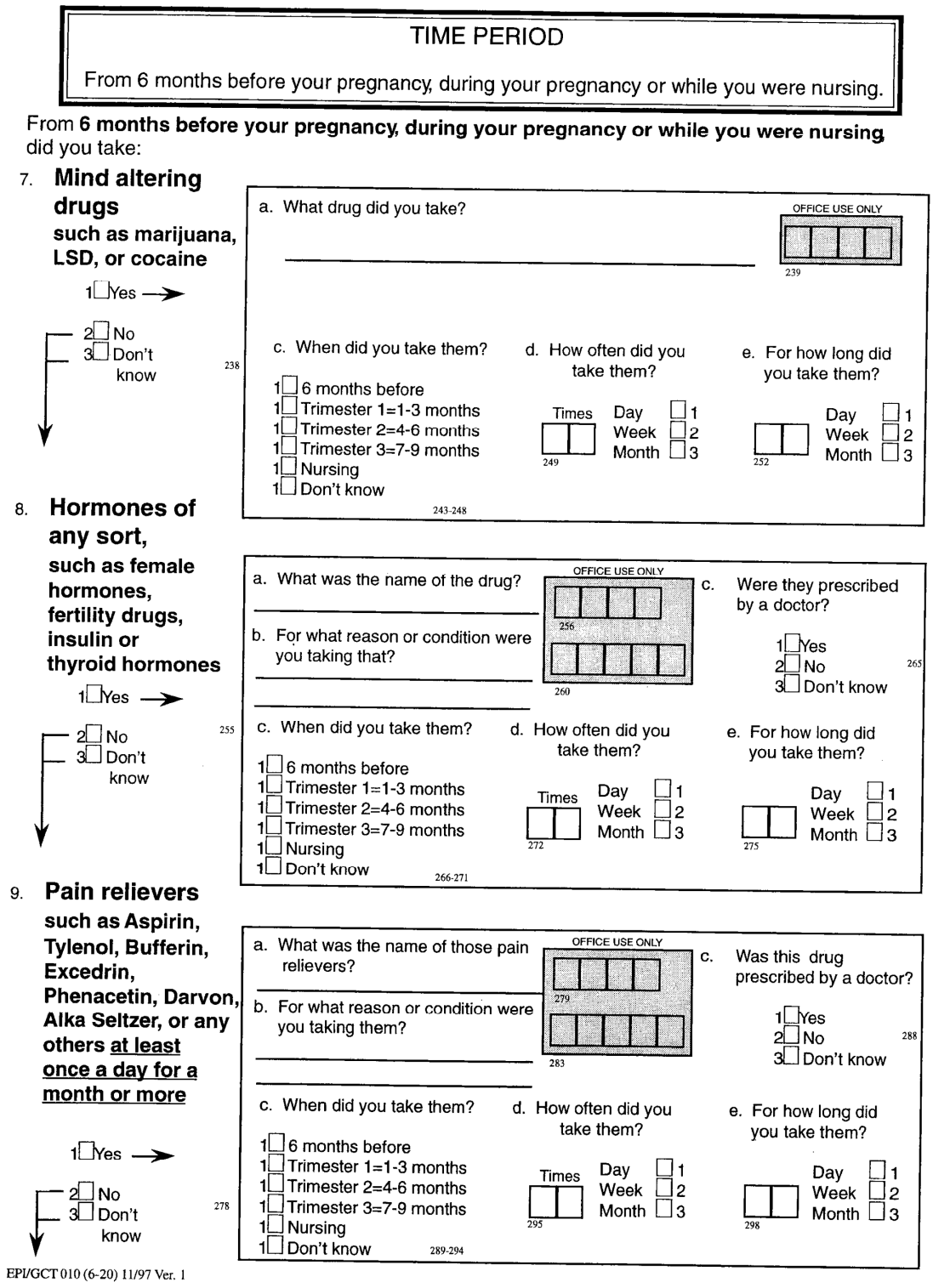
5 NO

8 DK

9 REF

GO TO SECTION E

Figure 4: GCT female hormones questions



Appendix B: Children's Oncology Group Institutions

Institutions with COG membership vary over time. The following lists provide the COG institution that opened AE24: Epidemiology of infant leukemia and AE22: Case-Control Study of Pediatric Germ Cell Tumors. Note that the institutions themselves may have changed over time, so the lists presented here do not represent current institutions associated with the COG.

Table 1: COG institutions that opened study AE24: Epidemiology of infant leukemia

Institution ID	Institution Name
3	Children's Hosp Med Ctr
5	Dell Children's Medical Center
61	Baystate Medical Center
67	Bellin Memorial Hospital
69	Cedars-Sinai Medical Ctr
71	Children's Hosp Cent Georgia
74	Children's Hosp of L.A.
76	Children's Hosp Orange Cty
78	Cincinnati Children's Hospital
79	Children's Hosp at Cleveland
81	City of Hope National Med Ctr
82	Nationwide Children's Hospital
87	Children's National Med Ctr-DC
88	Children's Hospital Denver
90	Dakota Midwest Cancer Inst
91	Children's Med Ctr Dayton
93	Henry Ford Hospital
95	Children's Hosp Central Calif.
99	Group Health Cooperative
100	Helen DeVos Children's Hosp
103	Miller Children's/Harbor-UCLA
104	Penn State Children's Hospital
106	Indiana University
113	Kalamazoo Ctr for Med Studies

114	Children’s Mercy Cancer Center
115	Children’s Hosp Kings Daughters
116	East Tennessee Children’s
117	Kosair Children’s Hospital
118	A.B. Chandler Med Center
119	Loma Linda Univ Med Ctr
121	Children's Hem/Onc Team
123	Lutheran Gen Child Med Ct
126	Mary Bridge Children’s Hospital
127	Mayo Clinic
129	A.I. duPont Hosp for Children
131	M.D. Anderson Cancer Ctr
133	MeritCare Medical Group
135	C.S. Mott Children’s Hospital
136	Univ of Minnesota Cancer Ctr.
137	Backus Children's Hospital
139	Mem Miller Children's Hospital
141	CancerCare Manitoba
143	Montefiore Medical Center
144	Children’s Hosp & Clinics of Mi
145	Marshfield Clinic
146	Memorial Sloan-Kettering
148	Michigan State University
150	Vanderbilt Children's Hospital
151	Kaiser Foundation Research Ins
155	IWK Health Centre
157	Schneider Children's No. Shore
159	Toledo Children's Hospital
160	Newark Beth Israel Medical Ctr
163	Children’s Hosp Oakland
164	Children’s Memorial Hosp Omaha
165	Children's Hosp, London Health
167	Children’s Hosp of Philadelphia
168	Phoenix Children’s Hosp
169	University of Pittsburgh
171	Doernbecher Children’s Hospital
175	Raymond Blank Children's Hosp.
176	Allan Blair Mem Clinic
177	South Carolina Cancer Center

178	Rainbow Babies Hospital
182	Methodist Children's Hospital
183	Saskatoon Cancer Center
184	Santa Barbara Cottage Hospital
185	So Calif Permanente
188	Seattle Children's
189	Southern Illinois U Sch of Med
196	St. Joseph's Hosp. & Med. Ctr.
197	Nevada Cancer Res Fdn CCOP
199	St. Vincent Children's Indiana
201	Tod Children’s Hosp.-Forum
205	Texas Tech UHSC - Amarillo
208	Connecticut Children's Med Ctr
209	University of Illinois
210	University of North Carolina
213	The Univ of Chicago Comer Chi
214	UCLA David Geffen School
215	UCSF School of Medicine
219	Primary Children’s Medical Ctr
220	New York Medical College
223	University of Wisconsin-AFCH
228	Child Hosp New Orleans/LSU CCO
232	Midwest Children's Cancer Ctr
233	Via Christi Regional Med Ctr
238	Oklahoma University
241	B.C.'s Children's Hospital
242	Baptist Children's Hospital
253	McMaster University
261	Tampa Children's Hospital
267	San Jorge Children's Hospital
272	Children's Atlanta, Emory Univ
274	Carilion Clinic Children’s Hosp
279	Child East Ontario
280	Cook Children's Medical Center
281	Advocate Hope Children's Hospi
282	Child Hosp Michigan
283	Dana-Farber Cancer Inst
286	Brody School of Med at ECU
288	Inova Fairfax Hospital

290	Stollery Children's Hospital
291	Baylor College of Medicine
293	All Children's Hosp
294	Child Mem Hosp (Chicago)
296	Carolinas Med Ctr
297	Child Hosp Greenville System
298	Hackensack Med Ctr
302	McGill Univ Health Ctr
303	Miami Children's Hosp
304	Hurley Medical Ctr
307	Mount Sinai Med Sch (N.Y.)
311	Nemours/Jacksonville
312	U of New Mexico
313	Rhode Island Hosp
314	Nemours/Orlando
316	St. Christopher's Hosp
319	Sacred Heart Hospital
320	Scott & White Memorial Hosp
321	St. Jude Children's Res. Hosp.
323	St John Hosp
327	Stanford University Med Ctr
328	SUNY Upstate Medical Univ
329	St. Vincent Hosp - Wisconsin
330	UT Southwestern Medical Center
331	Alberta Children's Hosp
334	UT/San Antonio
335	U of Alabama at Birmingham
336	U of Arkansas
338	UC/Davis
340	U of Florida
342	U of Kansas
347	Univ. of Missouri - Columbia
348	Eastern Maine Medical Center
349	U of Rochester
350	U of South Alabama
353	U of Vermont
354	Washington Univ
355	WV Univ, Charleston
357	Yale University

358	Rady Children's Hosp San Diego
359	Hosp Sick Children
362	Schneider Children's Hospital
363	Florida Hospital Cancer Inst.
364	Driscoll Children's Hosp
365	Legacy Emanuel Hospital and He
368	St. Mary's Hosp
369	U of Virginia
373	Wesley Med Ctr
374	Mission Hospitals
375	Medical City Children's Hosp
382	SUNY Stony Brook
384	Commonwealth Univ - MCV
385	Sanford Children’s Specialty
386	UMDNJ-Robert Wood Johnson Univ
387	Columbia Presbyterian College
391	Broward General Medical Center
454	Winthrop University Hospital
461	Sacred Heart Children's Hosp

Table 2: COG institutions that opened study AE22: Case-Control Study of Pediatric Germ Cell Tumors

Institution ID	Institution Name
3	Children's Hosp Med Ctr
4	Albany Medical Center
5	Dell Children's Medical Center
66	Downstate Medical Center
67	Bellin Memorial Hospital
68	Brookdale Hospital Medical Ctr
69	Cedars-Sinai Medical Ctr
74	Children's Hosp of L.A.
76	Children's Hosp Orange Cty
78	Cincinnati Children's Hospital
79	Children's Hosp at Cleveland
80	Columbia Medical Center - West
81	City of Hope National Med Ctr
82	Nationwide Children's Hospital
87	Children's National Med Ctr-DC
88	Children's Hospital Denver
90	Dakota Midwest Cancer Inst
91	Children's Med Ctr Dayton
93	Henry Ford Hospital
95	Children's Hosp Central Calif.
99	Group Health Cooperative
100	Helen DeVos Children's Hosp
101	Georgetown Univ Medical Ctr
103	Miller Children's/Harbor-UCLA
104	Penn State Children's Hospital
106	Indiana University
109	University of Iowa Hosp
111	Janeway Child Health Ctr
113	Kalamazoo Ctr for Med Studies
114	Children's Mercy Cancer Center
115	Children's Hosp Kings Daughters
116	East Tennessee Children's
117	Kosair Children's Hospital
119	Loma Linda Univ Med Ctr
126	Mary Bridge Children's Hospital
127	Mayo Clinic

129	A.I. duPont Hosp for Children
131	M.D. Anderson Cancer Ctr
133	MeritCare Medical Group
134	Clarian Health
135	C.S. Mott Children’s Hospital
136	Univ of Minnesota Cancer Ctr.
139	Mem Miller Children's Hospital
140	Morristown Memorial Hospital
143	Montefiore Medical Center
144	Children’s Hosp & Clinics of Mi
145	Marshfield Clinic
148	Michigan State University
150	Vanderbilt Children's Hospital
151	Kaiser Foundation Research Ins
155	IWK Health Centre
162	New York Univ Medical Center
163	Children’s Hosp Oakland
165	Children's Hosp, London Health
166	Atlantic Health System
167	Children’s Hosp of Philadelphia
168	Phoenix Children’s Hosp
169	University of Pittsburgh
171	Doernbecher Children’s Hospital
175	Raymond Blank Children's Hosp.
176	Allan Blair Mem Clinic
177	South Carolina Cancer Center
178	Rainbow Babies Hospital
182	Methodist Children's Hospital
183	Saskatoon Cancer Center
184	Santa Barbara Cottage Hospital
185	So Calif Permanente
188	Seattle Children's
189	Southern Illinois U Sch of Med
196	St. Joseph's Hosp. & Med. Ctr.
197	Nevada Cancer Res Fdn CCOP
199	St. Vincent Children's Indiana
202	Mercy Children's Hospital
205	Texas Tech UHSC - Amarillo
208	Connecticut Children's Med Ctr

209	University of Illinois
210	University of North Carolina
214	UCLA David Geffen School
215	UCSF School of Medicine
223	University of Wisconsin-AFCH
228	Child Hosp New Orleans/LSU CCO
238	Oklahoma University
241	B.C.'s Children's Hospital
253	McMaster University
272	Children's Atlanta, Emory Univ
279	Child East Ontario
283	Dana-Farber Cancer Inst
320	Scott & White Memorial Hosp
323	St John Hosp
342	U of Kansas
350	U of South Alabama
365	Legacy Emanuel Hospital and He
385	Sanford Children’s Specialty
386	UMDNJ-Robert Wood Johnson Univ

Appendix C: Latent Class Analysis Detail

An additional method for assessing infertility was explored for both the infant and the GCT study based on latent class analysis (LCA)[139]. This method combines variables that are each assumed to be informative of the exposure of interest (i.e. infertility). While each variable individually has the potential for misclassification of the true exposure of interest, using all the variables together may mitigate this misclassification. Both analyses included maternal age (continuous), history of infertility (ever tried for one year or more and not become pregnant) (yes/no), use of ovulation stimulating drugs before or during pregnancy (yes/no), history of multiple birth (yes/no), and history of recurrent pregnancy loss (<2, 2+). The infant study also included ever visit a doctor because it was difficult to get pregnant (yes/no), but this could not be included in the GCT study since it was not asked of everyone. Models with and without maternal age were also explored in order to determine if the effect of infertility was only through maternal age or if there was an independent risk factor for infertility apart from age.

The analysis used the conditional independence model, which assumes that the observed variables are independent of one another given class membership. This means that once infertility (as defined by the LCA model) is taken into account the observed variables used to measure infertility are not related to one another. The analysis maximizes the following likelihood:

$$\ell(\theta,\rho)=\prod_{i=1}^{n}\{\rho\prod_{k=1}^{K}P(Y_{ik}\mid E=1)+(1-\rho)\prod_{k=1}^{K}P(Y_{ik}\mid E=0)\}$$

Where E is the unobserved exposure of interest (E = 1 indicating infertility, E = 0 indicating normal fertility), K is the observed variables [Y_1, …,Y_K] for each subject i = 1, .., n, and ρ = the proportion where E = 1. Since more than three observed variables were used, the model was identifiable and no additional constraints were needed. LCA was conducted using M-Plus software[140]. Both two and three class models were fit to the data and model selection relied on the BIC value for model fit. Latent class membership was then determined based on the largest predicted class probability and used as a predictor in a logistic regression model along with potential confounders.

In both studies the two class model fit better than the three class model (Tables 1 and 2). Each two class model included an infertility and non-infertility group with higher proportions of each infertility indicator in the infertility group. In the infant study the infertility group included participants who sought medical advice for non-pregnancy into one group along with one additional participant who indicated use of ovulation stimulating drugs, but no medical advice for non-pregnancy. The classification did not change based on whether or not age was included in the model. The GCT study the infertility group was based on more of a mixture of variables and did change with the inclusion of maternal age with few included in the infertility class in the model without maternal age. The two class models with and without maternal age are presented in table 3.

Table 1: Comparison of two and three class LCA models in the infant leukemia study

		Two-class model		Three class model		
Variable	Observed	Class 1 (n = 638)	Class 2 (n = 129)	Class 1 (n = 129)	Class 2 (n = 331)	Class 3 (n = 307)
History of multiple birth (prior to index)						
No	0.98	0.99	0.93	0.93	0.98	1.00
Yes	0.02	0.01	0.07	0.07	0.02	0.00
Use of ovarian stimulating drugs						
No	0.96	1.00	0.75	0.73	1.00	1.00
Yes	0.04	0.00	0.25	0.27	0.00	0.00
Ever try for one year or more						
No	0.77	0.88	0.28	0.25	0.81	0.95
Yes	0.23	0.12	0.72	0.75	0.19	0.05
Ever visit a doctor or clinic for pregnancy						
No	0.83	1.00	0.06	0.03	0.98	1.00
Yes	0.17	0.00	0.94	0.97	0.02	0.00
Prior fetal loss						
None or one	0.94	0.95	0.86	0.86	0.92	0.98
Two or more	0.06	0.05	0.14	0.14	0.08	0.02
Maternal age						
mean	29.39	28.67	32.76	32.76	31.95	25.22
Fit Statistic						
BIC		6836.80		6855.38		

Table 2: Comparison of two and three class LCA models in the GCT study

		Two-class model		Three class model		
Variable	Observed	Class 1 (n = 57)	Class 2 (n = 644)	Class 1 (n = 223)	Class 2 (n = 392)	Class 3 (n = 86)
History of multiple birth (prior to index)						
No	0.97	0.94	0.97	0.96	0.98	0.94
Yes	0.03	0.06	0.03	0.04	0.02	0.06
Use of ovarian stimulating drugs						
No	0.98	0.80	1.00	0.99	1.00	0.79
Yes	0.02	0.20	0.00	0.01	0.00	0.21
Ever try for one year or more						
No	0.79	0.17	0.87	0.90	0.86	0.00
Yes	0.21	0.83	0.13	0.10	0.14	1.00
Prior fetal loss						
None or one	0.95	0.82	0.97	0.94	0.98	0.84
Two or more	0.05	0.18	0.03	0.06	0.02	0.16
Maternal age						
mean	27.26	31.39	26.73	30.63	23.86	31.78
Fit Statistic						
BIC		5712.66		5738.44		

Table 3: Comparison of two LCA models with and without maternal age in the GCT study

		With maternal age		Without maternal age	
Variable	Observed	Class 1 (n = 57)	Class 2 (n = 644)	Class 1 (n = 26)	Class 2 (n = 675)
History of multiple birth (prior to index)					
No	0.97	0.94	0.97	0.92	0.97
Yes	0.03	0.06	0.03	0.08	0.03
Use of ovarian stimulating drugs					
No	0.98	0.80	1.00	0.71	1.00
Yes	0.02	0.20	0.00	0.29	0.00
Ever try for one year or more					
No	0.79	0.17	0.87	0.00	0.85
Yes	0.21	0.83	0.13	1.00	0.15
Prior fetal loss					
None or one	0.95	0.82	0.97	0.79	0.96
Two or more	0.05	0.18	0.03	0.21	0.04
Maternal age					
mean	27.26	31.39	26.73		

Appendix D: Detailed recruitment procedures for the IMPART study

Study population

Two groups of children were recruited for this study; one born after ART treatment (ART group) and the other born after spontaneous conception (SC group). In the ART group, only women who conceived with fresh non-donor oocytes were eligible for inclusion of their children in this study. For SC children, the child must have been conceived without the use of fertility drugs or treatments. In the case of multiple births in either the ART or non-ART group only one child was selected for participation in the study. Children with phenotypic characteristics of BWS or AS or who had been diagnosed with imprinting disorders were excluded from the study.

A cohort of children born after ART was assembled by contacting patients with a reported successful pregnancy from the University of Minnesota Reproductive Medicine Center (UMRMC). The target age of children for this study was six months to five years. The records of mothers who reported a successful birth between March 2005 and December 2008 were accessed. These mothers were first contacted to participate through a letter sent by the UMRMC. After the letter was mailed, follow-up phone calls were made. The phone call introduced women to the study, answered any questions, and enrolled the women and her child into the study. Once verbal consent was given the participant either made an appointment at the Delaware Clinic Research Unit (DCRU) or requested a collection kit to take to a clinic located closer to her home. Up to 12 phone calls were made to initiate contact with potential participants. Calls varied by time of day

and day of the week to increase the chance of contact. On the 12th call a message was left requesting a return call. If no contact was made a second letter was mailed to encourage participation.

Children in the SC group were recruited from several different sources. The primary source of recruitment was through advertisements. Advertisements were placed around the University of Minnesota, at child care centers, and at pediatrician offices. In addition, fliers were distributed at the Minnesota State Fair, ART mothers were asked to recruit friends, and mailed advertisements were sent to patients seen at Fairview general pediatrics clinics. In the case of friend recruitment, friends were required to be contacted initially by the mother of the participating ART child and had to give permission for study staff to contact. Once permission was granted, information was sent through the mail followed by a phone call. In all other cases, potential subjects called or e-mailed study investigators and requested information through the mail. After the initial mailing (or e-mail) follow-up phone calls were conducted to determine interest in participating. Similar to the ART group, the phone call introduced the women to the study, answered any potential questions, and provided resources to set up an appointment for their child's blood and buccal cell donation through the DCRU or a mailed kit.

Different levels of consent were available to participants. Mothers could choose whether or not their child's sample could be stored for future research and whether or not we could contact them for future studies involving the stored samples.

Appendix E: IMPART study forms

Figure 1: ART group introductory letter

Date

«MotherName»
«Address»
«City», «State» «Zip»

Dear «MotherName»:

I am writing on behalf of the Reproductive Medicine Center and the Department of Pediatrics at the University of Minnesota. We are conducting the IMPART study, which will compare children conceived through assisted reproduction technology to children conceived spontaneously. We are contacting you because you gave birth to a child after treatment at the Reproductive Medicine Center.

If you agree to participate in this study we would ask you to complete a mailed questionnaire, release some of your medical records, and donate a sample of your child's blood and cheek cells. The questionnaire will ask about information related to your reproductive history, your pregnancy with your participating child, and about your and your child's vitamin use. We will also ask for your permission to obtain medical records from your treatment at the Reproductive Medicine Center as well as your prenatal and delivery records.

We will also ask to obtain a blood sample and a cheek brush sample from your child to obtain genetic material to study *DNA methylation and imprinting*. DNA methylation and imprinting are involved in turning genes on and off. There is not a lot known about what variation in DNA methylation means, but some studies give us reason to think it may be different in some children conceived by ART. If you are willing, your child's DNA will be collected through a blood draw and cheek swab performed at a research clinic at the University of Minnesota by a trained nurse or certified medical assistant or, alternatively, through a mailed collection kit which you would take to your local clinic to have the samples collected.

Additionally, you would have the option to help us recruit spontaneously conceived children by contacting your friends with a child around the same age as your child.

The results of this study may help us determine whether children born after assisted reproductive technology could have different DNA methylation, but we wish to emphasize that we won't be sure what the results may mean for your child.

Susan Puumala, the study coordinator, will be calling you in the next several days to answer your questions and ask if you want to participate, or you may call her directly at 612-624-0162. The enclosed consent form also has information about the IMPART study. We would ask that you please read the entire form and sign it if you would like to participate.

Sincerely,

Mark Damario, MD, FACOG

Figure 2: SC group introductory letter

Date

«MotherName»
«Address»
«City», «State» «Zip»

Dear «MotherName»:

Thank you for your interest in the study we are conducting through the Department of Pediatrics at the University of Minnesota. The study is called the IMPART study and will compare children conceived through assisted reproduction technology to children conceived spontaneously. Below is a brief outline of the study detailing what would be required of you and your child.

If you agree to participate in this study we would ask you to complete a mailed questionnaire, donate a sample of your child's blood and cheek cells, and release your prenatal and delivery medical records. The questionnaire will ask about information related to your reproductive history, your pregnancy with your participating child, and about your and your child's vitamin use.

The blood and cheek brush sample from your child will be used to obtain genetic material to study *DNA methylation and imprinting*. DNA methylation and imprinting are involved in turning genes on and off. There is not a lot known about what variation in DNA methylation means, but some studies give us reason to think it may be different in some children conceived by ART. If you are willing, your child's DNA will be collected through a blood draw and cheek swab performed at a research clinic at the University of Minnesota by a trained nurse or certified medical assistant or, alternatively, through a mailed collection kit which you would take to your local clinic to have the samples collected.

The results of this study may help us determine whether children born after assisted reproductive technology could have different DNA methylation, but we wish to emphasize that we won't be sure what the results may mean for your child.

Susan Puumala, the study coordinator, will follow-up with you in the next several days to answer your questions and ask if you want to participate, or you may call her directly at 612-624-0162. The enclosed consent form also has information about the IMPART study. We would ask that you please read the entire form, sign it, and return it along with the questionnaire and medical records release form if you would like to participate.

Sincerely,

Logan G. Spector, PhD

Figure 3: ART group consent form

CONSENT FORM

The Imprinting and Methylation Patterns after Assisted Reproductive Technology (IMPART) Study

Your child is invited to participate in a research study which will compare the DNA of children who were conceived by assisted reproductive technology (ART) to those who were conceived without assistance. You are being asked to allow your child to participate because you gave birth to a child after being treated at the University of Minnesota Reproductive Medicine Center. Please read this form and ask any questions you may have before agreeing to let your child participate.

The IMPART Study is being conducted by researchers in the Department of Pediatrics, the Division of Epidemiology and Community Health, and the Reproductive Medicine Center at the University of Minnesota. The researchers involved in this study include Logan G. Spector, Ph.D., Heather Nelson, Ph.D., Mark Damario, MD, and Susan Puumala, MS.

Study Purpose

The purpose of the IMPART study is to examine a process called DNA methylation in children. DNA methylation is important in turning genes on and off. There is not a lot known about what variation in DNA methylation means, but some studies give us reason to think it may be different in some children conceived by ART. We are therefore investigating DNA methylation patterns in children conceived with and without the help of ART.

Study Procedures

If you agree to participate in this study, we would ask you to do the following:

- *Donate a sample of your child's blood and cheek cells.* You will need to schedule a visit to the General Clinic Research Center at the University of Minnesota for your child's appointment or request a kit to take to your local clinic. A trained nurse or certified medical assistant will collect 5-10 milliliters (1-2 teaspoons) of your child's blood. Cheek cells will be collected at the clinic visit using a soft brush. We will use the blood and cheek cells to obtain DNA to test methylation.

- *Fill out a questionnaire about yourself and your reproductive history.* The questions will ask about your reproductive history, your pregnancy with your participating child, and some information about your and your child's vitamin use. It takes about 10 minutes to complete. This questionnaire will be mailed to you to fill out at home and send back to us.

- *Grant permission to access your University of Minnesota Reproductive Medicine Center records.* We will ask you to sign a separate authorization for this if you agree to participate in the study.

- *Grant permission to access prenatal and delivery medical records.* We will ask you to sign a separate authorization for this if you agree to participate in the study.

- *Optionally, contact friends with children about the same age as your child to participate in the study.* If you choose, we would ask you to contact your friends who might be interested in the study and have a child about the same age as your child. If they are interested in the study you would then forward their contact information to the study coordinator.

Since it is not known how differences in DNA methylation and imprinting might influence future health the results will not provide useful information to you or your child. This information is for research purposes only and you will not be informed of your individual results. However, if you wish, you may be informed of the overall findings from the study once it is over.

All samples will be kept in our laboratory at the University of Minnesota labeled with only an ID number. Individuals will not be identified in any publication or reports of this data.

Sample storage and future contact

Some of the genes we will be looking at in the IMPART study are known now, but we might also think of things to look at later. Because of this, with your permission we would like to store any left over samples we collect for a period of ten years.

At the end of this form you will be asked to initial your choice about storing samples and future contact. The choices are:

- Allow us to keep your child's sample after the initial tests and allow us to contact you about future studies. If you agree to this option, we will keep your name and other contact information in our database.

- Allow us to keep your child's sample after the initial tests but do not allow us to contact you about future studies. If you agree to this option, we will remove your name and other contact information from our database after the initial testing is done, within 2 years after enrollment in the study.

- Have us destroy your child's sample after the initial testing is done. If you agree to this option, we will remove your names and other contact information from our database after the initial testing is done and destroy your child's sample within 2 years after enrollment in the study.

You can change your mind later about storing samples. If you do change your mind, please call the researchers to let them know.

Risks of Study Participation

There are a few risks of study participation. Blood collection has the risk of temporary pain and possible bruising at the collection site as well as a small chance of infection. To protect your child against these risks only qualified, well trained nurses or certified medical assistants will collect the blood. Another potential risk of participation is that your personal information could accidentally be released to someone other than study staff. To protect against this risk we keep all personal information in locked file cabinets or in computer databases protected by passwords. All study samples are kept in our laboratory at the University of Minnesota labeled only with an ID number.

Benefits of Study Participation

There will be no direct benefit to you or your child. This research may help us understand different patterns of gene expression in children.

Study Costs/Compensation

There is no cost to you for participating in this study. We will give you a Target gift card worth $25 for participation in the study and pay for your parking when you come to the clinic for your child's blood donation.

Confidentiality

The records of this study will be kept private. In any publications or presentations, we will not include any information that will make it possible to identify you or your child as a subject. Your record for the study may, however, be reviewed by departments at the University with appropriate regulatory oversight. Your participation in this study will not be noted in your medical record. To these extents, confidentiality is not absolute.

Protected Health Information (PHI)

Your PHI created or received for the purposes of this study is protected under the federal regulation known as HIPAA. Refer to the attached HIPAA authorization for details concerning the use of this information.

Voluntary Nature of the Study

Participation in this study is voluntary. Your decision whether or not to participate in this study will not affect your current or future relations with the University of Minnesota, the University of Minnesota Physicians or the Reproductive Medicine Center. If you decide to participate, you are free to withdraw at any time without affecting those relationships.

Contacts and Questions

The researchers conducting this study are Logan G. Spector, Susan Puumala, and their associates in the Cancer Center and Reproductive Medicine Center at the University of Minnesota. You may ask any questions you have now, or if you have questions later, **you are encouraged to** contact them at 612-624-0162.

If you have any questions or concerns regarding the study and would like to talk to someone other than the researcher(s), **you are encouraged to** contact the Fairview Research Helpline at telephone number 612-672-7692 or toll free at 866-508-6961. You may also contact this office in writing or in person at Fairview University Medical Center - Riverside Campus, #815 Professional Building, 2450 Riverside Avenue, Minneapolis, MN 55454.

You will be given a copy of this form to keep for your records.

Options for Sample Storage and Future Contact

We would like to keep left over samples for future laboratory research. You can decide not to allow this. Please put your initials by your decision below:

_____ I agree to have my child's left over samples stored for future laboratory research.

_____ I agree to have my child's left over samples stored for future laboratory research, but do not want to be contacted about participating in future studies.

_____ I do not agree to have my child's left over samples stored for future laboratory research. I would like my child's left over samples destroyed once the initial tests are done, within 2 years after enrollment in the study.

If you change your mind about your choice later, you can call the researchers to let them know.

Statement of Consent

I have read the above information. I have asked questions and have received answers. I consent to participate in the study.

Signature of Subject_____

Date_____

Figure 4: SC group consent form

CONSENT FORM

The Imprinting and Methylation Patterns after Assisted Reproductive Technology (IMPART) Study

Your child is invited to participate in a research study which will compare the DNA of children who were conceived by assisted reproductive technology (ART) to those who were conceived without assistance. You are being asked to allow your child to participate because you were contacted by a friend about the study or you answered a study advertisement. Please read this form and ask any questions you may have before agreeing to let your child participate.

The IMPART Study is being conducted by researchers in the Department of Pediatrics, the Division of Epidemiology and Community Health, and the Reproductive Medicine Center at the University of Minnesota. The researchers involved in this study include Logan G. Spector, Ph.D., Heather Nelson, Ph.D., Mark Damario, MD, and Susan Puumala, MS.

Study Purpose

The purpose of the IMPART study is to examine a process called DNA methylation in children. DNA methylation is important in turning genes on and off. There is not a lot known about what variation in DNA methylation means, but some studies give us reason to think it may be different in some children conceived by ART. We are therefore investigating DNA methylation patterns in children conceived with and without the help of ART.

Study Procedures

If you agree to participate in this study, we would ask you to do the following:

- *Donate a sample of your child's blood and cheek cells.* You will either need to schedule a visit to the General Clinic Research Center at the University of Minnesota for your child's appointment or request a kit to take to your local clinic. A trained nurse or certified medical assistant will collect 5-10 milliliters (1-2 teaspoons) of your child's blood. Cheek cells will be collected at the clinic visit using a soft brush. We will use the blood and cheek cells to obtain DNA to test methylation.

- *Fill out a questionnaire about yourself and your reproductive history.* The questions will ask about your reproductive history, your pregnancy with your participating child, and some information about your and your child's vitamin use. It takes about 10 minutes to complete. This questionnaire will be mailed to you to fill out at home and send back to us.

- *Grant permission to access prenatal and delivery medical records.* We will ask you to sign a separate authorization for this if you agree to participate in the study.

Since it is not known how differences in DNA methylation and imprinting might influence future disease the results will not provide useful information to you or your child. This information is for research purposes only and you will not be informed of your individual results. However, if you wish, you may be informed of the overall findings from the study once it is over.

All samples will be kept in our laboratory at the University of Minnesota labeled with only an ID number. Individuals will not be identified in any publication or reports of this data.

Sample storage and future contact

Some of the genes we will be looking at in the IMPART study are known now, but we might also think of things to look at later. Because of this, with your permission we would like to store any left over samples we collect for a period of ten years.

At the end of this form you will be asked to initial your choice about storing samples and future contact. The choices are:

- Allow us to keep your child's sample after the initial tests and allow us to contact you about future studies. If you agree to this option, we will keep your name and other contact information in our database.

- Allow us to keep your child's sample after the initial tests but do not allow us to contact you about future studies. If you agree to this option, we will remove your name and other contact information from our database after the initial testing is done, within 2 years after enrollment in the study.

- Have us destroy your child's sample after the initial testing is done. If you agree to this option, we will remove your names and other contact information from our database after the initial testing is done and destroy your child's sample within 2 years after enrollment in the study.

You can change your mind later about storing samples. If you do change your mind, please call the researchers to let them know.

Risks of Study Participation

There are a few risks of study participation. Blood collection has the risk of temporary pain and possible bruising at the collection site as well as a small chance of infection. To protect your child against these risks only qualified, well trained nurses or certified medical assistants will collect the blood. Another potential risk of participation is that your personal information could accidentally be released to someone other than study staff. To protect against this risk we keep all personal information in locked file cabinets or in computer databases protected by passwords. All study samples are kept in our laboratory at the University of Minnesota labeled only with an ID number.

Benefits of Study Participation

There will be no direct benefit to you or your child. This research may help us understand different patterns of gene expression in children.

Study Costs/Compensation

There is no cost to you for participating in this study. We will give you a Target gift card worth $25 for participation in the study and pay for your parking when you come to the clinic for your child's blood donation.

Confidentiality

The records of this study will be kept private. In any publications or presentations, we will not include any information that will make it possible to identify you or your child as a subject. Your record for the study may, however, be reviewed by departments at the University with appropriate regulatory oversight. Your participation in this study will not be noted in your medical record. To these extents, confidentiality is not absolute.

Protected Health Information (PHI)

Your PHI created or received for the purposes of this study is protected under the federal regulation known as HIPAA. Refer to the attached HIPAA authorization for details concerning the use of this information.

Voluntary Nature of the Study

Participation in this study is voluntary. Your decision whether or not to participate in this study will not affect your current or future relations with the University of Minnesota. If you decide to participate, you are free to withdraw at any time without affecting those relationships.

Contacts and Questions

The researchers conducting this study are Logan G. Spector, Susan Puumala, and their associates in the Cancer Center and Reproductive Medicine Center at the University of Minnesota. You may ask any questions you have now, or if you have questions later, **you are encouraged to** contact them at 612-624-0162.

If you have any questions or concerns regarding the study and would like to talk to someone other than the researcher(s), **you are encouraged to** contact the Fairview Research Helpline at telephone number 612-672-7692 or toll free at 866-508-6961. You may also contact this office in writing or in person at Fairview University Medical Center - Riverside Campus, #815 Professional Building, 2450 Riverside Avenue, Minneapolis, MN 55454.

You will be given a copy of this form to keep for your records.

Options for Sample Storage and Future Contact

We would like to keep left over samples for future laboratory research. You can decide not to allow this. Please put your initials by your decision below:

_____ I agree to have my child's left over samples stored for future laboratory research.

_____ I agree to have my child's left over samples stored for future laboratory research, but do not want to be contacted about participating in future studies.

_____ I do not agree to have my child's left over samples stored for future laboratory research. I would like my child's left over samples destroyed once the initial tests are done, within 2 years after enrollment in the study.

If you change your mind about your choice later, you can call the researchers to let them know.

Statement of Consent

I have read the above information. I have asked questions and have received answers. I consent to participate in the study.

Signature of Subject_____

Date_____

Figure 5: ART group study questionnaire

The Imprinting and Methylation Patterns after Assisted Reproductive Technology (IMPART) Study

Maternal Questionnaire

Thank you for taking part in the University of Minnesota IMPART study. The purpose of this study is to examine possible difference in epigenetic patterns between children conceived through assisted reproduction technology and children conceived spontaneously.

The study includes four parts: (1) filling out this survey, (2) giving us sample of your child's blood and cheek cells, (3) giving us permission to access your medical records at the University of Minnesota Reproductive Medicine Center as well as your prenatal and deliver medical records, and (4) optionally, contacting friends with children about the same age as your child to participate in the study.

This survey asks for general information about you as well as information about your reproductive history, your pregnancy with your participating child, and some information about your and your child's vitamin use. It will take about 10 minutes to fill out. Please answer every question as best you can. If you do not know the answer to a question, please provide your best guess.

In order to obtain the most valid information possible, **it is important that this survey is answered by the person to whom it was addressed**.

If you have any other questions as you fill out this survey, please contact the study coordinator, Susan Puumala, at (612) 624-0162 or puumala@umn.edu.

Section 1: Question about your participating child

A1. What is your participating child's date of birth?

___ ___ / ___ ___ / ___ ___ ___ ___
month day year

A2. According to the doctor's due date, was your participating child born more than 6 days early, more than 6 days late, or about on time?

☐ Early
☐ On time
☐ Late
☐ Don't know

A3. What was your participating child's birth weight?

☐☐ (POUNDS) ☐☐ (OUNCES)

A4. How old were you when you became pregnant with your participating child?

☐☐ Years old

A5. How old was the child's father when you became pregnant with your participating child?

☐☐ Years old

A6. Was your participating child a single birth, or did you have twins or triplets?

☐ Single
☐ Twins
☐ Triplets
☐ Quadruplets or higher

A7. What gender is your participating child?

☐ Male

☐ Female

A8. Does your participating child have any of the following conditions? (Mark all that apply)

☐ Cleft lip or palate

☐ Spina bifida or other spinal defect

☐ Down syndrome

☐ Turner's syndrome

☐ Klinefelter's syndrome

☐ Any kidney, bladder, or sex organ abnormality

☐ Any heart defect

☐ Other birth defect, please specify _____

☐ None

A9. Was your participating child ever breastfed or fed breast milk?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

A10. How old was your child when he/she completely stopped breastfeeding or being fed breast milk?

☐ Three months or younger

☐ Between three and six months

☐ Between six months and one year

☐ One year or more

☐ Currently still breastfeeding

Section 2: Health and Pregnancy History

B1. Are you currently pregnant?

☐ Yes

☐ No

B2. How many times have you been pregnant altogether, including live births, stillbirths, miscarriages, abortions, molar pregnancies and tubal or ectopic pregnancies (excluding current pregnancy)?

☐☐ Times

B3. How many pregnancies resulted in a live birth?

☐☐ Number

B4. Please list the month and year of each live birth and circle the number of children born at each delivery.

Birth 1:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 2:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 3:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 4:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 5:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 6:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**

B5. How many pregnancies resulted in a stillbirth?

☐☐ Number

B6. How many pregnancies resulted in a miscarriage?

☐☐ Number

B7. How many pregnancies resulted in an abortion?

☐☐ Number

B8. How many pregnancies resulted in a molar pregnancy?

☐☐ Number

B9. How many pregnancies resulted in a tubal or ectopic pregnancy?

☐☐ Number

B10. Were you diagnosed with cancer prior to the birth of your participating child?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

B11. What type of cancer was it?

- ☐ Breast
- ☐ Colon or Rectal
- ☐ Leukemia
- ☐ Lymphoma
- ☐ Endometrial
- ☐ Thyroid
- ☐ Lung
- ☐ Melanoma
- ☐ Other skin cancer
- ☐ Other cancer

B12. What type of treatment did you receive for your cancer? (mark all that apply)

- ☐ Surgery
- ☐ Chemotherapy
- ☐ Radiation
- ☐ None
- ☐ Other

B13. Was the child's father diagnosed with cancer prior to the birth of your participating child?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

B14. What type of cancer was it?

- ☐ Prostate
- ☐ Testicular
- ☐ Colon or Rectal
- ☐ Leukemia
- ☐ Lymphoma
- ☐ Thyroid
- ☐ Lung
- ☐ Melanoma
- ☐ Other skin cancer
- ☐ Other cancer

B15. What type of treatment did he receive for his cancer? (mark all that apply)

- ☐ Surgery
- ☐ Chemotherapy
- ☐ Radiation
- ☐ None
- ☐ Other

Section 3: Vitamin Supplementation

C1. During your pregnancy with your participating child, did you take a multivitamin supplement?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C2. Was it prescribed by a doctor?

☐ No

☐ Yes

C3. How many days per week did you usually take this multivitamin?

☐ Less than 1 day per week

☐ 1 – 3 days per week

☐ 4 – 5 days per week

☐ 6 – 7 days per week

☐ Not sure

C4. During your pregnancy with your participating child did you take any other vitamin supplements besides a multivitamin?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C5. Please list all the other vitamin supplements you took during your pregnancy.

_____

_____

_____

_____

C6. Does your participating child currently take a multivitamin?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C7. How many days per week does your child usually take this multivitamin?

☐ Less than 1 day per week

☐ 1 – 3 days per week

☐ 4 – 5 days per week

☐ 6 – 7 days per week

☐ Not sure

Section 4: Demographics

D1. Are you of Hispanic or Latino origin?

☐ No

☐ Yes

D2. Which best describes your racial background (choose the <u>one</u> you most identify with)?

☐ White

☐ Black or African American

☐ Native Hawaiian or other Pacific Islander

☐ Asian

☐ American Indian/Alaska Native

☐ Other, please specify: _____

D3. What is your marital status?

☐ Married or living in a marriage-like relationship

☐ Widowed

☐ Divorced

☐ Separated

☐ Never married

D4. What is the <u>highest</u> level of schooling you have completed? (Mark one.)

☐ 8th grade or less

☐ Some high school

☐ High school graduate

☐ GED (high school equivalency)

☐ 1 to 3 years vocational education beyond high school

☐ Some college

☐ College graduate

☐ One or more years of graduate or professional school

D5. Which of the following best describes your <u>total</u> household income?

☐ Up to $10,000
☐ More than $10,000 up to $20,000
☐ More than $20,000 up to $40,000
☐ More than $40,000 up to $60,000
☐ More than $60,000 up to $80,000
☐ More than $80,000 up to $100,000
☐ More than $100,000

Thank you for your time!

Figure 6: SC group study questionnaire

The Imprinting and Methylation Patterns after Assisted Reproductive Technology (IMPART) Study

Maternal Questionnaire

Thank you for taking part in the University of Minnesota IMPART study. The purpose of this study is to examine possible difference in epigenetic patterns between children conceived through assisted reproduction technology and children conceived spontaneously.

The study includes three parts: (1) filling out this survey, (2) giving us sample of your child's blood and cheek cells, and (3) giving us permission to access your prenatal and delivery medical records.

This survey asks for general information about you as well as information about your reproductive history, your pregnancy with your participating child, and some information about your and your child's vitamin use. It will take about 10 minutes to fill out. Please answer every question as best you can. If you do not know the answer to a question, please provide your best guess.

In order to obtain the most valid information possible, **it is important that this survey is answered by the person to whom it was addressed**.

If you have any other questions as you fill out this survey, please contact the study coordinator, Susan Puumala, at (612) 624-0162 or puumala@umn.edu.

Section 1: Question about your participating child

A1. What is your participating child's date of birth?

___ ___ / ___ ___ / ___ ___ ___ ___
month day year

A2. According to the doctor's due date, was your participating child born more than 6 days early, more than 6 days late, or about on time?

☐ Early
☐ On time
☐ Late
☐ Don't know

A3. What was your participating child's birth weight?

☐☐ (POUNDS) ☐☐ (OUNCES)

A4. How old were you when you became pregnant with your participating child?

☐☐ Years old

A5. How old was the child's father when you became pregnant with your participating child?

☐☐ Years old

A6. Was your participating child a single birth, or did you have twins or triplets?

☐ Single
☐ Twins
☐ Triplets
☐ Quadruplets or higher

A7. What gender is your participating child?

☐ Male

☐ Female

A8. Does your participating child have any of the following conditions? (Mark all that apply)

☐ Cleft lip or palate

☐ Spina bifida or other spinal defect

☐ Down syndrome

☐ Turner's syndrome

☐ Klinefelter's syndrome

☐ Any kidney, bladder, or sex organ abnormality

☐ Any heart defect

☐ Other birth defect, please specify _____

☐ None

A9. Was your participating child ever breastfed or fed breast milk?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

A10. How old was your child when he/she completely stopped breastfeeding or being fed breast milk?

☐ Three months or younger

☐ Between three and six months

☐ Between six months and one year

☐ One year or more

☐ Currently still breastfeeding

A11. At the time you became pregnant with your participating child were you trying to get pregnant?

☐ No ⟶ Go to question the top of the next page.

☐ Yes

↓

A12. How long had you been trying?

☐ Less than one year

☐ One year or more

Section 2: Health and Pregnancy History

B1. Are you currently pregnant?

☐ Yes

☐ No

B2. How many times have you been pregnant altogether, including live births, stillbirths, miscarriages, abortions, molar pregnancies and tubal or ectopic pregnancies (excluding current pregnancy)?

☐☐ Times

B3. How many pregnancies resulted in a live birth?

☐☐ Number

B4. Please list the month and year of each live birth and circle the number of children born at each delivery.

Birth 1:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 2:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 3:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 4:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 5:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**
Birth 6:	☐☐	**Month**	☐☐☐☐	**Year**	**Total children this delivery?**	**1**	**2**	**3**	**4**	**5+**

B5. How many pregnancies resulted in a stillbirth?

☐☐ Number

B6. How many pregnancies resulted in a miscarriage?

☐☐ Number

B7. How many pregnancies resulted in an abortion?

☐☐ Number

B8. How many pregnancies resulted in a molar pregnancy?

☐☐ Number

B9. How many pregnancies resulted in a tubal or ectopic pregnancy?

☐☐ Number

B10. Were you diagnosed with cancer prior to the birth of your participating child?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

B11. What type of cancer was it?

☐ Breast
☐ Colon or Rectal
☐ Leukemia
☐ Lymphoma
☐ Endometrial
☐ Thyroid
☐ Lung
☐ Melanoma
☐ Other skin cancer
☐ Other cancer

B12. What type of treatment did you receive for your cancer? (mark all that apply)

☐ Surgery
☐ Chemotherapy
☐ Radiation
☐ None
☐ Other

B13. Was the child's father diagnosed with cancer prior to the birth of your participating child?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

B14. What type of cancer was it?

- ☐ Prostate
- ☐ Testicular
- ☐ Colon or Rectal
- ☐ Leukemia
- ☐ Lymphoma
- ☐ Thyroid
- ☐ Lung
- ☐ Melanoma
- ☐ Other skin cancer
- ☐ Other cancer

B15. What type of treatment did he receive for his cancer? (mark all that apply)

- ☐ Surgery
- ☐ Chemotherapy
- ☐ Radiation
- ☐ None
- ☐ Other

Section 3: Vitamin Supplementation

C1. During your pregnancy with your participating child, did you take a multivitamin supplement?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C2. Was it prescribed by a doctor?

☐ No

☐ Yes

C3. How many days per week did you usually take this multivitamin?

☐ Less than 1 day per week

☐ 1 – 3 days per week

☐ 4 – 5 days per week

☐ 6 – 7 days per week

☐ Not sure

C4. During your pregnancy with your participating child did you take any other vitamin supplements besides a multivitamin?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C5. Please list all the other vitamin supplements you took during your pregnancy.

_____

_____

_____

_____

C6. Does your participating child currently take a multivitamin?

☐ No ⟶ Go to top of next page.

☐ Yes

↓

C7. How many days per week does your child usually take this multivitamin?

☐ Less than 1 day per week

☐ 1 – 3 days per week

☐ 4 – 5 days per week

☐ 6 – 7 days per week

☐ Not sure

Section 4: Demographics

D1. Are you of Hispanic or Latino origin?

☐ No

☐ Yes

D2. Which best describes your racial background (choose the <u>one</u> you most identify with)?

☐ White

☐ Black or African American

☐ Native Hawaiian or other Pacific Islander

☐ Asian

☐ American Indian/Alaska Native

☐ Other, please specify: _____

D3. What is your marital status?

☐ Married or living in a marriage-like relationship

☐ Widowed

☐ Divorced

☐ Separated

☐ Never married

D4. What is the <u>highest</u> level of schooling you have completed? (Mark one.)

☐ 8th grade or less

☐ Some high school

☐ High school graduate

☐ GED (high school equivalency)

☐ 1 to 3 years vocational education beyond high school

☐ Some college

☐ College graduate

☐ One or more years of graduate or professional school

D5. Which of the following best describes your <u>total</u> household income?

☐ Up to $10,000
☐ More than $10,000 up to $20,000
☐ More than $20,000 up to $40,000
☐ More than $40,000 up to $60,000
☐ More than $60,000 up to $80,000
☐ More than $80,000 up to $100,000
☐ More than $100,000

Thank you for your time!

Appendix F: IMPART study detailed lab methods

Pyrosequencing background

Methylation analysis was performed using pyrosequencing techniques. Pyrosequencing uses ATP to produce a reaction with individual nucleotides enabling changes in DNA sequences to be detected. Specifically, four enzymes are used to cause a chemical reaction as each nucleotide is sequentially incorporated. The reaction caused by these enzymes results in luminescence that is used to assess the pyrophosphate release as each nucleotide is incorporated[238]. If a particular nucleotide is incorporated into the sequence then light is emitted and the intensity of the light signal is captured, while unincorporated nucleotides are degraded.[239] The resulting light signals are then displayed in a pyrogram that provides information about the sequence.

For the methylation analysis, gDNA was treated with sodium bisulfite, resulting in a conversion of unmethylated cytosines to uracils. PCR was used to amplify the region of interest on the bisulfite converted DNA. During this PCR uracil is replaced with thymine resulting in a C/T polymorphism. Pyrosequencing then detects methylation by examining the artificially created as a C/T polymorphism. However, unlike a normal SNP, there is a quantitative level of the C/T polymorphism. Thus the level of methylation is calculated as the light signal from the methylated peak over the total light signal for the C/T pair[240].

IMPART study lab methods

Sample collection and storage

Blood and buccal cell collection was performed through two different mechanisms. Samples were collected on campus through the DCRC. For participants not able to come to campus for the sample collection, kits were mailed and collection was performed at a local clinic. Up to 6mL of blood was collected through a venous blood draw. The buccal cell sample was collected from cheek cells using two Catch-All™ Sample Collection Swabs (Epicentre, Madison, WI, USA).

Buccal cell processing

DNA was extracted from buccal swabs using the Purgene DNA isolation method (Qiagen, Germantown/Gaithersburg, USA). The specific steps are outlined below:

1. In 1.5mL microcentrifuge tube, add 600uL Puregene® Cell Lysis Solution and 30uL Proteinase K solution to swab and incubate at 55°C for ≥1 hour.
2. Add 3uL RNase A solution to swab and incubate at 37°C for ≥15 minutes.
3. Remove swab and add 200uL Protein Precipitation Solution to lysate, and incubate on ice for ≥5 minutes.
4. Centrifuge samples for 5 minutes at 13000rpm.
5. Collect supernatant and add it to 600uL Isopropanol and 1uL Glycogen solution and incubate at -20°C overnight.
6. Centrifuge samples for 10 minutes at 13000rpm.
7. Discard supernatant and wash DNA pellet with 600uL 70% ethanol.

8. Vacuum dry DNA pellet and rehydrate with 30uL low-EDTA TE buffer at 65°C for ≥1 hour.

Blood sample processing

Initial processing was performed in the lab of Dr. Julie Ross. Frozen samples were transferred to the lab of Dr. Heather Nelson for DNA extraction and pyrosequencing. Initial processing took place within 24 hours of sample collection (although some samples did not have time stamp, so it is not clear if more than 24 hours had elapsed prior to processing). Details of the initial processing are below:

1. Collect blood sample from clinic or FedEx mailing.
2. In an RNase-free environment, dilute blood 1:2 with PBS, and layer up to 5mL over Ficoll in 15mL centrifuge tube. Repeat with a new tube for every ~5mL diluted blood.
3. Centrifuge at 1500 rpm for 30 minutes to separate cells.
4. Remove top plasma layer and aliquot into 1.5mL microcentrifuge tubes. Store at -80°C.
5. Gently remove all buffy coats (lymphocyte layers), transfer to one clean 15mL tube, and raise volume to 10mL with PBS. Divide the dilute lymphocytes equally in two 15mL tubes.
6. Discard Ficoll and red blood cells.
7. Centrifuge lymphocytes at 2000 rpm for 10 minutes to pellet. Discard supernatant.

8. Resuspend one pellet with 3mL RNAprotect Cell Reagent and divide equally into three 1.5mL microcentrifuge tubes. Store at -80°C.
9. Resuspend one pellet with 3mL PBS and divide equally into three 1.5mL microcentrifuge tubes. Snap freeze in ethanol/dry ice bath. Store at -80°C.

DNA for methylation analysis was extracted from the lymphocyte pellet without the RNAprotect cell reagent. DNA extraction steps using QIAamp DNA Mini kit spin protocol for blood and body fluids (Qiagen, Germantown/Gaithersburg, USA) are listed below:

1. Pipet 20 µl proteinase K into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl lymphocytes to the microcentrifuge tube.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside of the lid.
6. Add 200 µl ethanol to the sample and mix again by pulse- vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20000 x g; 14000 rpm) for 3 min.
10. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE. Incubate at room temperature for 1 min and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Genetic regions analyzed

In this study, three regions of interest were examined for differential methylation IGF2/H19, KvDMR, and IGF2R. These regions were selected based on their association with BWS and their association with cancer. Specifically, we analyzed IGF DMR0 (3 CpGs), 3rd (11 CpGs) and 6th (16 CpGs) CTCF-binding site of H19 DMR, IGF2R (15 CpGs), and KvDMR (7 CpGs). The 5th CpG cite in the 6th CTCF-binding site of H19 DMR is known to be polymorphic and so was excluded from the analysis[230].

Methylation analysis

Methylation analysis was performed using pyrosequencing techniques. The process was the same for all sites analyzed. Genomic DNA was isolated from the blood samples and treated with sodium bisulfite using the EZ methylation kit (Zymo research). Primers and procedures for IGF DMR0, 3rd and 6th CTCF-binding site of H19 DMR, and IGF2R were the same as in Boissonnas, et al[230]. Briefly, 1μL bisulfite treated DNA was combined with 1x HotStar Taq buffer that was supplemented with 1.6μM MgCl2, 100 μM dNTPs, 2 U HotStar Taq polymerase and 5 pmol of forward and reverse primers with one of the primers biotinylated. Denaturing was performed at 95°C for 15 minutes followed by 50 cycles of 30 s at 95°C, annealing was performed at specific temperatures for each loci for 30 s and 20s at 72°C, the extension step was performed for 5 min at 72°C. The protocol for the KvDMR region was different and based on Bourque, et al[233]. After bisulfite conversion, PCR was performed using 1x HotStar Taq buffer, 0.2 mM dNTP, 5 pmol of each primer with one of the primers biotinylated, 1.0 U HotStar Taq polymerase, and 2μL bisulfite converted DNA. Denaturing was performed at 95°C for 10 minutes followed by 40 cycles of 40 s at 95°C, annealing was carried out at 55°C for 40 s and 40s at 72°C, the extension step was performed for 7 min at 72°C. After PCR amplification, pyrosequencing was performed for all regions using the PyroMark MD system and analyzed using the accompanying software (Qiagen, Germantown/Gaithersburg, USA). PCR and pyrosequencing primers are provided in table 1.

Several quality assurance checks are performed by the pyrosequencing software through examining the sequence obtained with the sequence expected. There are six specific checks to assess the quality of the pyrosequencing run. The signal to noise ratio is examined to determine if enough signal is present for an accurate read. Three related checks compare the peaks produced by the pyrogram to the expected peaks from the sequence. Peaks are examined for correct height and width. In addition, a similar check assesses whether or not the total signal from C/T pair is equal to the total peak height expected. A peak height or width that is different from what is expected could indicate a deviation from the sequence or a problem reading the sequence. Another check examines the pyrogram for split peaks. A split peak suggests a problem when incorporating a nucleotide into the sequence. Finally, consistency among the CpG sites in each region of interest is examined to identify potential problems at a particular site. The software returns an overall assessment of quality: pass, uncertain or fail. All samples with an assessment of uncertain or fail were examined and either a technical problem with a non-CpG site was found or the sample was retested until it passed all quality assurance measures.

Table 1: PCR and sequencing primers

Region	PCR primers	Sequencing primer	#CpGs	Sequence length
IGF DMR0	Forward: TATTTATTTAGGGTGGTGTT Reverse: TCCAAACACCCCCACCTTAA	Primer 1: TTTTTAGGAAGTATAGTTA	3	19
H19 DMR CTCF3	Forward: TTGGTAGGTATAGAAATTGGGG Reverse: ACACCTAACTTAAATAACCCAAAA	Primer 1: GTAGTATATGGGTATTTGTG Primer 2: GTGGATTTAAAAGTGGT	3 8	49 84
H19 DMR CTCF6	Forward: TGGGTATTTTTGGAGGTTTTTTT Reverse: ATAAATATCCTATTCCCAAATAA	Primer 1: TTTATYGTTTGGATGG Primer 2: GTAGGTTTATATATTATAG Primer 3: GTTTYGGGTTATTTAAGTTA	6 6 4	50 49 20
IGF2R	Forward: TTTTGAAAAGGAGGTAGAAAAAGGT Reverse: TCTTAAAAACTAACTAAAAACCTAAC	Primer 1: GGAAGGGAGTGATTTAGT Primer 2: AAGGGTTAGGTAGGGAT	12 3	45 74
KvDMR	Forward: TTAGTTTTTTGYGTGATGTGTTTATTA Reverse: CCCACAAACCTCCACACC	Primer 1: TTGYGTGATGTGTTTATTA	7	58

Appendix G: Additional analysis for the IMPART study

Sensitivity analysis

Sensitivity analysis was conducted to examine the effect of retested samples on the outcome of the methylation analysis. Retested samples failed quality assurance checks on the first run and were later retested. Although all samples retested passed quality assurance checks, there appeared to be a large difference between these samples and the originally run samples. Table 1 gives the overall results without the retested samples included.

Overall, the results did not change whether or not the retested samples were included in the analysis. This suggests that retested samples are not different enough from the first run samples to have an impact on the results.

Table 1: Multiple regression model results without retest samples

Gene region	ART group Mean*	SC group Mean*	Difference Estimate**	95% CI	p-value
Lymphocytes					
KvDMR	46.11	45.23	0.88	(-0.90, 2.66)	0.33
H19 CTCF3	43.91	42.81	1.10	(-0.73, 2.92)	0.24
H19 CTCF6	33.85	33.47	0.38	(-2.67, 3.43)	0.81
IGF2DMR0	48.78	49.12	-0.35	(-2.35, 1.66)	0.73
IGF2R	69.70	72.81	-3.11	(-8.78, 2.56)	0.28
Buccal cells					
KvDMR	50.70	49.90	0.80	(-3.57, 5.17)	0.72
H19 CTCF3	42.73	72.75	-0.02	(-2.86, 2.81)	0.99
H19 CTCF6	35.95	34.19	1.76	(-3.18, 6.70)	0.48
IGF2DMR0	36.94	34.57	2.37	(-0.14, 4.89)	0.06
IGF2R	78.74	81.74	-3.00	(-3.00, 1.66)	0.07

*The reported mean is averaged over all CpG sites in the region of interest
**Adjusted for child's age, child's birth weight, child's gender, maternal age, maternal education, and household income

Full model results

The following tables present the estimates for the full models for each analysis including not only the ART group results, but also the parameter estimates for the cofounding variables included.

Table 2: Multiple regression results for KvDMR lymphocyte samples

Parameter	Estimate	SE	p-value
Intercept	42.00	4.01	<.0001
Group			
ART vs. Non-ART	0.96	0.91	0.29
Batch			
Initial vs. 3rd batch	6.82	2.21	0.002
2nd batch vs. 3rd batch	4.75	2.17	0.03
Child's age			
Years	0.11	0.38	0.77
Child's gender			
Female vs. Male	0.40	0.80	0.62
Child's birth weight			
500g	0.28	0.21	0.18
Maternal education			
< college degree vs. advanced education	-0.84	1.36	0.54
college degree vs. advanced education	1.12	0.96	0.24
Maternal age at birth			
Years	-0.09	0.10	0.41
Income			
<40K vs. 80K+	2.33	0.96	0.02
40K-<80K vs. 80K+	0.002	0.81	0.998

Table 3: Multiple regression results for KvDMR buccal samples

Parameter	Estimate	SE	p-value
Intercept	59.74	8.38	<.0001
Group			
ART vs. Non-ART	1.89	2.33	0.42
Batch			
Initial vs. other	-3.93	1.77	0.03
Child's age			
Years	1.83	0.94	0.05
Child's gender			
Female vs. Male	-2.16	2.02	0.28
Child's birth weight			
500g	-0.01	0.68	0.99
Maternal education			
< college degree vs. advanced education	-1.99	2.41	0.41
college degree vs. advanced education	1.40	2.30	0.54
Maternal age at birth			
Years	-0.31	0.22	0.15
Income			
<40K vs. 80K+	-1.79	2.80	0.52
40K-<80K vs. 80K+	-2.35	1.94	0.22

Table 4: Multiple regression results for IGF2 DMR0 lymphocyte samples

Parameter	Estimate	SE	p-value
Intercept	46.26	5.31	<.0001
Group			
ART vs. Non-ART	-0.11	1.00	0.91
Batch			
Initial vs. other	2.88	0.82	0.0004
Child's age			
Years	-0.58	0.48	0.22
Child's gender			
Female vs. Male	2.72	0.85	0.001
Child's birth weight			
500g	0.14	0.24	0.55
Maternal education			
< college degree vs. advanced education	0.74	1.39	0.60
college degree vs. advanced education	1.03	0.93	0.27
Maternal age at birth			
Years	0.01	0.14	0.93
Income			
<40K vs. 80K+	-0.74	1.15	0.52
40K-<80K vs. 80K+	-0.33	0.93	0.73

Table 5: Multiple regression results for IGF2 DMR0 buccal samples

Parameter	Estimate	SE	p-value
Intercept	33.60	4.69	<.0001
Group			
ART vs. Non-ART	2.33	1.23	0.06
Batch			
Initial vs. other	0.94	1.37	0.49
Child's age			
Years	-0.04	0.62	0.95
Child's gender			
Female vs. Male	3.01	1.10	0.006
Child's birth weight			
500g	0.67	0.34	0.049
Maternal education			
< college degree vs. advanced education	-4.50	1.60	0.005
college degree vs. advanced education	1.22	1.26	0.33
Maternal age at birth			
Years	-0.18	0.11	0.12
Income			
<40K vs. 80K+	3.50	2.05	0.09
40K-<80K vs. 80K+	1.12	1.18	0.34

Table 6: Multiple regression results for H19 CTCF3 lymphocyte samples

Parameter	Estimate	SE	p-value
Intercept	40.90	2.79	<.0001
Group			
ART vs. Non-ART	0.90	0.82	0.27
Batch			
Initial vs. 4th batch	-3.24	0.75	<.0001
2nd batch vs. 4th batch	-2.19	1.72	0.20
3rd batch vs. 4th batch	-3.03	0.93	0.001
Child's age			
Years	0.39	0.33	0.24
Child's gender			
Female vs. Male	0.66	0.65	0.31
Child's birth weight			
500g	0.10	0.15	0.51
Maternal education			
< college degree vs. advanced education	-1.63	1.06	0.13
college degree vs. advanced education	-0.56	0.72	0.44
Maternal age at birth			
Years	0.06	0.07	0.44
Income			
<40K vs. 80K+	1.22	1.06	0.25
40K-<80K vs. 80K+	-0.42	0.71	0.55

Table 7: Multiple regression results for H19 CTCF3 Buccal samples

Parameter	Estimate	SE	p-value
Intercept	48.65	6.27	<.0001
Group			
ART vs. Non-ART	0.20	1.43	0.89
Batch			
Initial vs. 4th batch	-0.84	1.19	0.48
2nd batch vs. 4th batch	-0.45	1.20	0.71
3rd batch vs. 4th batch	1.20	0.75	0.11
Child's age			
Years	1.05	0.66	0.11
Child's gender			
Female vs. Male	-1.18	1.30	0.36
Child's birth weight			
500g	-0.02	0.48	0.97
Maternal education			
< college degree vs. advanced education	-2.30	1.72	0.18
college degree vs. advanced education	-0.16	1.61	0.92
Maternal age at birth			
Years	-0.19	0.15	0.19
Income			
<40K vs. 80K+	-1.79	2.80	0.52
40K-<80K vs. 80K+	-2.35	1.94	0.22

Table 8: Multiple regression results for H19 CTCF6 lymphocyte samples

Parameter	Estimate	SE	p-value
Intercept	36.36	5.43	<.0001
Group			
ART vs. Non-ART	1.09	1.43	0.45
Batch			
Initial vs. 3rd batch	2.18	0.33	<.0001
2nd batch vs. 3rd batch	-4.51	1.03	<.0001
Child's age			
Years	0.08	0.63	0.89
Child's gender			
Female vs. Male	1.99	0.94	0.03
Child's birth weight			
500g	0.17	0.26	0.51
Maternal education			
< college degree vs. advanced education	0.83	1.78	0.64
college degree vs. advanced education	0.32	1.09	0.77
Maternal age at birth			
Years	-0.08	0.15	0.58
Income			
<40K vs. 80K+	-0.69	1.62	0.67
40K-<80K vs. 80K+	-0.96	1.24	0.44

Table 9: Multiple regression results for H19 CTCF6 buccal samples

Parameter	Estimate	SE	p-value
Intercept	43.88	8.36	<.0001
Group			
ART vs. Non-ART	2.05	2.24	0.36
Batch			
Initial vs. 3rd batch	-5.36	1.49	0.0003
2nd batch vs. 3rd batch	-5.52	3.04	0.07
Child's age			
Years	1.79	0.84	0.03
Child's gender			
Female vs. Male	-0.89	1.90	0.64
Child's birth weight			
500g	0.22	0.72	0.76
Maternal education			
< college degree vs. advanced education	-4.68	2.25	0.04
college degree vs. advanced education	0.24	2.34	0.92
Maternal age at birth			
Years	-0.21	0.20	0.30
Income			
<40K vs. 80K+	-2.21	2.06	0.28
40K-<80K vs. 80K+	-1.89	1.96	0.33

Table 10: Multiple regression results for IGF2R lymphocyte samples

Parameter	Estimate	SE	p-value
Intercept	83.67	11.56	<.0001
Group			
ART vs. Non-ART	-4.32	2.65	0.10
Batch			
Initial vs. 3rd batch	8.12	5.64	0.15
2nd batch vs. 3rd batch	5.17	2.46	0.04
Child's age			
Years	-1.02	1.40	0.47
Child's gender			
Female vs. Male	-1.19	2.19	0.59
Child's birth weight			
500g	0.33	0.53	0.53
Maternal education			
< college degree vs. advanced education	-7.44	2.59	0.004
college degree vs. advanced education	0.17	2.40	0.95
Maternal age at birth			
Years	-0.35	0.23	0.13
Income			
<40K vs. 80K+	1.75	3.09	0.57
40K-<80K vs. 80K+	-1.17	1.86	0.53

Table 11: Multiple regression results for IGF2R buccal samples

Parameter	Estimate	SE	p-value
Intercept	92.77	6.86	<.0001
Group			
ART vs. Non-ART	-2.83	1.56	0.07
Batch			
Initial vs. 3rd batch	-2.99	1.34	0.03
2nd batch vs. 3rd batch	-0.51	1.54	0.74
Child's age			
Years	-1.12	0.71	0.11
Child's gender			
Female vs. Male	0.13	1.07	0.90
Child's birth weight			
500g	-0.25	0.32	0.43
Maternal education			
< college degree vs. advanced education	-1.41	1.81	0.43
college degree vs. advanced education	1.88	1.14	0.10
Maternal age at birth			
Years	-0.12	0.16	0.47
Income			
<40K vs. 80K+	-3.17	1.74	0.07
40K-<80K vs. 80K+	-1.99	1.07	0.06