**STATISTICAL ANALYSIS PLAN FOR BIRTH DEFECTS FROM
IMPAIRED GLUCOSE METABOLISM**

1.   Background and Hypothesis Development

1.1   Introduction

The risk of congenital anomalies in offspring is higher among women who have type 1 or type 2 diabetes before or during the early stages of pregnancy (Becerra, Khoury, Corder, & Erickson, 1990). Major congenital malformations are the leading cause of mortality and serious morbidity in infants of mothers with established diabetes (Kitzmiller, Buchanan, Kjos, Combs, & Ratner, 1996). Animal models show the teratogenicity of impaired glucose metabolism throughout the range of elevated glucose, not just for the most extreme values that in humans would be called frank diabetes (Moley, Vaughn, & Diamond, 1994). However, the current literature offers no definitive findings regarding the association between lesser magnitudes of glucose intolerance and birth defects in humans. It is important to fill this gap in the understanding of human fetal development because impaired glucose tolerance (IGT) is more common than frank diabetes, so that, even if relative risks for IGT are smaller than those for frank diabetes, the population disease burden (e.g., as measured by population attributable risk) may be higher for IGT. Another reason this issue is important is that the prevalences of both IGT and pregestational type 2 diabetes are rising along with the obesity epidemic in the United States. Further, many studies looking at the effects of impaired glucose metabolism in early pregnancy on congenital anomalies have examined only known cases of diabetes, thus failing to include women who have unrecognized or undiagnosed forms of impaired glucose metabolism in analyses. (Farrell, Neale, & Cundy, 2002; Schaefer et al., 1997; Anderson et al., 2005; Kanwar et al., 2005).

In summary, while there is strong evidence of the impact of diagnosed pre-existing and gestational diabetes on pregnancy outcomes, there is inadequate information about the influence of impairment of glucose tolerance. To address this issue, the following broad hypothesis has been developed for investigation using data collected in the National Children's Study (NCS):

> Impaired glucose metabolism during pregnancy is associated with increased risk of major congenital malformations of the heart, central nervous system, musculoskeletal system, and all congenital birth defects combined.

1.2   Prevalence of Birth Defects and of Impaired Glucose Metabolism During Pregnancy

A complication in studying congenital birth defects is that there are many forms of congenital malformations, all of which are relatively rare. The three most common birth defects in the United States are major congenital malformations of the heart (0.6 percent of births) (Hoffman & Kaplan, 2002); of the central nervous system (0.3 percent of births) (Branum & Schoendorf, 2003); and of the musculoskeletal system (0.2 percent of births) (Feuchtbaum et al., 1999). Overall, an estimated 3-4 percent of all births in the United States exhibit one or more congenital malformations (Leppig, Werler, Cann, Cook, & Holmes, 1987; Lynberg & Edmonds, 1994).

IGT is defined as two-hour glucose levels of 140 to 199 mg/dL on the 75-g oral glucose tolerance test (OGTT), and impaired fasting glucose is defined as glucose levels of 100 to 125 mg/dL in fasting patients (Rao, Disraeli, & McGregor, 2004). Prevalence estimates of IGT differ depending on the test used for its diagnosis. Based on data from the 1999-2002 National Health and Nutrition Examination Survey (NHANES), approximately 26 percent of women between 20 and 39 years old have impaired

fasting glucose, 1.7 percent have diagnosed diabetes, and 0.7 percent have undiagnosed diabetes (Cowie et al., 2006). However, since pregnancy is known to increase the risk of IGT, the prevalence of IGT within the NCS sample of pregnant women will be higher. It has been estimated that about 4-7 percent of pregnancies in the United States are complicated by gestational diabetes, with possibly higher rates in some ethnic groups (Kjos & Buchanan, 1999).

1.3 Development of a Specific Hypothesis

In a clinical setting, IGT is commonly measured by using either fasting glucose alone, a fasting OGTT, or a combination of the two. While these measures would give the most accurate determination of a pregnant woman's impaired glucose metabolism, the NCS has decided for practical reasons not to collect fasting glucose measures in early pregnancy. Participant burden issues related to the unpleasantness of these tests for pregnant women and the need to retain the women and their offspring in the Study over many years has led to a decision to rely on a less burdensome test based on non-fasting blood samples collected during the first trimester of pregnancy. These samples will be used to assess Hemoglobin A1c (HgbA1c) levels, which do not depend on the fasting state of the woman.

The level of HgbA1c, also known as glycated hemoglobin or glycosylated hemoglobin, is an indicator of the person's blood sugar control during the previous 2-3 months. HgbA1c is formed when glucose in the blood binds irreversibly to hemoglobin to form a stable molecule. It possesses the lifespan of normal red blood cells (90-120 days), being eliminated from the bloodstream only when the red cells are replaced. Several studies have demonstrated that HgbA1c levels are directly proportional to the concentration of glucose in the blood over the full lifespan of the red blood cells and are not subject to the fluctuations that are seen with daily blood glucose monitoring (Shibata et al., 2005; Davidson, Schriger, Peters, & Lorber, 1999; Hassan, Johnson, Nader, Gannon, & Nuttall, 2006; Woerle et al., 2004). In healthy individuals, HgbA1c makes up approximately 4-6 percent of all hemoglobin in the bloodstream, with values as high as 12 percent in those individuals with poorly controlled diabetes. The standard of care guidelines for diabetics is to maintain the HgbA1c level at 7 percent or less (American Diabetes Association, 2007); however, it has been shown that risk of coronary heart disease increases with HgbA1c levels above 4.6 percent (Khaw et al., 2004; Selvin et al., 2005). In a study of women who required insulin therapy, HgbA1c levels measured in the first trimester were found to have a strong association with adverse pregnancy outcomes, with a value of 7.8 mg/dL in women whose children had major malformations vs. 6.7 mg/dL in the others (Schaefer et al., 1997). These results support the use of HgbA1c as an effective indicator of IGT in situations where fasting blood glucose samples cannot be obtained.

Since the adverse effects of IGT for organogenesis do not appear to be limited to specific organ systems, the primary hypothesis examines all congenital birth defects grouped together as one outcome. The primary hypothesis presented above is therefore operationalized for use with NCS data to the more specific hypothesis that is the main basis of the statistical analysis plan presented in this document:

Elevated HgbA1c levels during the first trimester of gestation are associated with increased risk of congenital birth defects.

A secondary hypothesis concerns the association between IGT and congenital heart defects, which are the most prevalent organ system birth defects. The NCS sample size will be large enough to provide sufficient statistical power to detect sizable effects for this subset of birth defects. As a result, the following secondary hypothesis is proposed:

> Elevated HgbA1c levels during the first trimester of gestation are associated with increased risk of congenital heart defects.

1.4   Justification for This Analysis

Three aspects of the NCS make it an extremely good source of data for analyzing the specific hypothesis identified above:

- The analyses will be able to assess the effects of HgbA1c levels on a nationally representative sample of live births as well as provide national estimates of the distribution of HgbA1c levels measured in the first trimester.

- The very large NCS sample size will result in a large number of birth defects for the analysis.

- The NCS longitudinal design will provide the standardized data needed to quantify HgbA1c levels in the first trimester of pregnancy and birth defects that may be detected up to age 2 (see below).

2.   The Variables Used in the Analysis

This section describes the variables that will be used in the analyses of the specific hypotheses relating first trimester HgbA1c levels and congenital birth defect outcomes, which have been discussed in Section 1 above. The section starts with precise descriptions of the methods that will be used to measure the outcome variable, i.e., presence or absence of congenital birth defects (Section 2.1), and follows with descriptions of the HgbA1c exposure variable (Section 2.2) and the covariates that will be used in the analyses (Section 2.3).

2.1   Outcome Definitions and Measurement

Definitions of Outcomes

All major congenital malformations diagnosed in live-born infants by 24 months of age will be included in this analysis (see Appendix I of the Research Plan for a complete list of diagnostic measures). There are two primary outcomes to be analyzed: (1) any major congenital malformation (approximate prevalence 3-4 percent); and (2) major congenital heart defects (approximate prevalence 0.6 percent) (Hoffman & Kaplan, 2002).

NCS field staff and photo reviewers will assess presence of major congenital malformations based on the NCS protocol. NCS research staff will use such measures as a 12-14 week ultrasound and direct examination occurring at birth, the 6-month visit, and the 12-month visit to diagnose various congenital malformations. Staff will also collect medical records in support of diagnoses of malformations reported by parents on questionnaires through 2 years of age. Utilizing these data, the NCS

will identify such birth defects as facial clefts, ear abnormalities, other head/facial abnormalities, skin and hair abnormalities, limb abnormalities, hand and foot abnormalities, congenital heart defects, chest wall abnormalities, abdominal wall defects, gastrointestinal defects, neural tube defects, urogenital malformations, and major chromosomal disorders. Reviews of medical records and maternal interview data will be used to detect the existence of birth defects that are not identified by photo review, as in the case of cardiac birth defects.

A few live-born infants will die before they reach the age of 2 years. A number of those infants who die soon after birth are expected to have birth defects. If the infant dies before hospital discharge, hospital discharge records will not be available. In an effort to identify such cases, the NCS will attempt to obtain death certificates from which to collect data regarding the presence of birth defects. Infants for whom the death certificates indicate the presence of a major congenital birth defect will be included as cases in the analysis. Those infants who die prior to discharge and whose death certificate and autopsy report do not indicate that a birth defect was present at the time of death will be recorded as not having a birth defect.

To reach a final determination and classification of a congenital birth defect, a panel of medical experts will adjudicate all reported birth defects through review of medical records and images, including sonograms. This adjudication process will also include a review of all death certificate and autopsy reports associated with each live birth.

2.2   Definition and Measurement of Exposure

Standard blood samples will be collected during the scheduled first trimester visit for all pregnant women who have been enrolled in the NCS by that time and have consented to provide the blood samples. Sample volumes will be kept to a minimum to reduce the burden to the pregnant women. Following collection of these biospecimens, they will be analyzed and/or stored in one or more repositories for future analysis. Since these blood samples will be used for a number of different investigations, their use for the current analyses must be carefully controlled.

Following the 2-year observation period during which congenital malformation diagnoses will be made for the NCS children, all children with birth defects (cases) and a sample of children without birth defects (controls) will be selected for the current analyses. If selected, an aliquot of the mother's stored first-trimester blood sample will be used to analyze the HgbA1c level. HgbA1c levels will be measured as a percentage, which may be categorized for some analyses.

2.3   Definition and Measurement of Covariates

A critical consideration in analyzing the association between first trimester HgbA1c levels and congenital birth defects is the inclusion of relevant covariates in the analyses. Potential confounders that are causally prior to the first trimester HgbA1c level must be incorporated in the analyses since they may explain away any association observed between HgbA1c levels and birth defects. Also potential effects modifiers (or interaction effects) that result in differential levels of association need to be included in order to provide meaningful interpretation of the associations under different conditions. The many important covariates that will be considered in the statistical analyses are listed below. For convenience they are grouped by type. The determination of which of these covariates are confounders and which are effect modifiers will be made based on theoretical considerations and the statistical methods described in sections 4.3 and 4.4.

2.3.1 Covariates to be Investigated

2.3.1.1 Covariates Related to Individual and Family History of Morbidity

Previous history of birth defects and/or family history of birth defects: Mothers whose first child had a birth defect are 2.4 times more likely than other women to have a second infant with a birth defect. Most of the risk is accounted for by the same defect recurring (Lie, Wilcox, & Skjaerven, 1994). Perhaps one of the strongest covariates in the association between impaired glucose metabolism and congenital birth defects is a family history of birth defects, spontaneous abortions, stillbirths, and subfertility (Jenkins et al., 2007). About 20 percent of birth defects are hereditary, resulting from the interaction of genes from one or both parents plus environmental influences. Defects may include cleft lip and palate, spina bifida, and heart defects (Shaw, Rozen, Finnell, Wasserman, & Lammer, 1998; Lott, 1996).

Pre-pregnancy and pregnancy body mass index (BMI): Obesity prior to and during pregnancy, which is a result of a complex interaction between many variables related to diet and physical activity, is associated with an increased risk of birth defects and with impaired glucose metabolism (Anderson et al., 2005). Prepregnancy obesity is associated with an increased risk for birth defects (Waller et al., 2007) and may modify the impact of glucose metabolism on the risk of congenital birth defects (Hotamisligil, 2006). It will be analyzed as both a categorical variable (underweight, normal, overweight, obese, morbidly obese) and as a continuous variable.

Previous history of gestational diabetes: A woman who experienced gestational diabetes in a previous pregnancy is at an increased risk of recurrent gestational diabetes in subsequent pregnancies and at an increased risk of type 2 diabetes mellitus in her lifetime. Birth defects have been associated with gestational diabetes, which probably reflects an association with undiagnosed type 2 diabetes.

2.3.1.2 Covariates Related to Demographic Factors

Race/ethnicity: Racial and/or ethnic factors are often associated with both variation in prevalence of birth defects (Correa, McCarter, Downing, Ferencz, & the Baltimore-Washington Infant Study Group, 1991; Canfield et al., 2006) and variation in the risk of IGT among women of reproductive age.

Socioeconomic status (SES): Because access to quality health services can be associated with the risk of IGT as well as with variation in the prevalence of birth defects, SES needs to be taken into account in an evaluation of IGT and risk of birth defects.

Area of residence: Women who reside in the same geographic area are exposed to the same environmental risk factors. Previous research has demonstrated an association between environmental air quality and both impaired first trimester glucose tolerance and congenital birth defects in offspring (Gilboa et al., 2005).

2.3.1.3 Covariates Related to Behavior/Lifestyle Factors

Smoking status: Nicotine and carbon monoxide play a role in causing adverse pregnancy outcomes (U.S. Department of Health and Human Services, 2004; Law et al., 2003; American College of Obstetricians and Gynecologists, 2000; Wang et al., 2002; Little, Cardy, & Mungar, 2004). Cigarette smoke has been shown to reduce the efficacy of folic acid in the prevention of congenital birth defects

(Shaw, Nelson, et al., 2002). Smoking is also associated with an increased risk for oral clefts, but the evidence for an association with other defects is limited and inconsistent. Whether smoking is associated with IGT is not clear.

Use of medication: Anticonvulsants can cause serious problems, including mental retardation and slow growth, in the developing fetus. Other drugs associated with birth defects include antipsychotic and antianxiety agents and certain antibiotics (Jones, 1996; Hernandez-Diaz, Werler, Walker, & Mitchell, 2000, 2001). There is also some evidence that some antidepressants may be associated with birth defects and with exacerbation of IGT, and that some antibiotics with a folate-antagonist effect can increase the risk of certain defects.

Physical activity: An increase in physical activity either prior to or during the first weeks of pregnancy is a recommended course of action for women who present with impaired glucose metabolism, both to improve glucose metabolic function and to reduce the risk of adverse health effects on the offspring from prenatal IGT (Jenkins et al., 2007).

Time of entry into prenatal care: Time of entry into prenatal care may serve as a proxy measure for quality of care for IGT and use of medications for glucose metabolism. Receipt of such care to manage impaired glucose metabolism either prior to or during pregnancy through behavior, diet, medication, etc., may affect a mother's glucose metabolism as well as overall nutritional status.

Use of nutritional supplements: Supplementation with folic acid attenuates the risk for neural tube defects (Berry et al., 1999; Bower & Stanley, 1989; Czeizel, 1993; Czeizel & Dudas, 1992; Daly, Kirke, Molloy, Weir, & Scott, 1995; Laurence, James, Miller, Tennant, & Campbell, 1981; Milunsky et al., 1989; MRC Vitamin Study Research Group, 1991), cardiac defects (Botto, Khoury, Mulinare, & Erickson, 1996; Botto, Mulinare, & Erickson, 2000; Czeizel, 1996: Czeizel, Toth, & Rockenbauer, 1996; Lewis, Van Dyke, Stumbo, & Berg, 1998; Shaw, Nelson, et al., 2002), oral clefts (Itikala, Watkins, Mulinare, Moore, & Liu, 2001; Lewis et al., 1998; Shaw, Lammer, Wasserman, O'Malley, & Tolarova, 1995; Tolarova & Harris, 1995; Yang, Khoury, Olney, & Mulinare, 1997), and urinary tract defects (Czeizel, 1996; Lewis et al., 1998; Werler, Hayes, Louik, Shapiro, & Mitchell, 1999; Yang et al., 1997). Supplementation with antioxidant vitamins appears to attenuate risk for cardiac defects (Correa, Botto, Liu, Mulinare, & Erickson, 2003).

Drug use: Recreational drug use in pregnant women has been associated with arm and leg abnormalities and central nervous system problems in offspring (Jones, 1996). Whether use of recreational drugs affects the risk of IGT is unclear.

2.3.1.4 Covariates Related to Intrinsic Factors

Cardiometabolic risk factors: The presence and level of cardiometabolic risk factors (e.g., obesity, hypertension, dyslipidemia, C-reactive protein, and other markers of low-grade inflammation), which in turn are related to behavioral factors such as diet and physical activity, in a pregnant woman's bloodstream have been shown to be associated with impaired glucose metabolism (Hotamisligil, 2006). Some of these factors have also been shown to be associated with risk of birth defects.

Lipid profile: Maternal fat-modified diets result in lower total and HDL cholesterol in infants and may be a suitable way to prevent cardiovascular disease among infants from the beginning of life (Fard, Mehrabian, Sarraf-Zadegan, & Sajadi, 2004). Central adiposity, which may be difficult to measure or detect, is associated with increased serum triglycerides and cholesterol levels, which are associated with an increased risk for IGT.

Serum inositol: The mechanisms by which hyperglycemia leads to birth defects are not clear but are probably complex and could be related to oxidative stress, low inositol levels, and other metabolic abnormalities associated with hyperglycemia. Studies in animals suggest low levels of inositol may be associated with an increased risk of neural tube defects (NTDs) and other defects (Cockroft, Brook, & Copp, 1992; Hashimoto et al., 1990; Baker, Piddington, Goldman, Egler, & Moehring, 1990; Green & Copp, 1997; Cogram et al., 2002). A recent case-control study also suggests that low serum levels of inositol may be associated with an increased risk for spina bifida (Groenen et al., 2003). Thus, in evaluating the role of hyperglycemia, it is important to take into account the inositol status of women early in pregnancy. Inositol, a sugar with important structural and functional properties, is part of the diet but is also synthesized by the body.

Gene-nutrient interactions: Methylenetetrahydrafolate reductase (MTHFR) polymorphisms appear to be associated with increased risk of birth defects (Botto & Yang, 2000). Reduced folate carrier and MTHFR appear to interact with folic acid supplementation to modify the risk of birth defects (Shaw, Nelson, et al., 2002; Shaw et al., 1998). However, there is no evidence to suggest that these gene-nutrient interactions have any association with IGT.

Parity and multiple births: The number of fetuses present in a pregnancy may be associated with risk of birth defects (greater risk among multiples than among singletons). However, whether multiple births are associated with IGT is not clear. While parity or number of births has been not been shown to be a confounder of the association between glucose metabolism and birth defects , parity of births does play a significant role in other birth outcomes, such as low birth weight.

Age of mother: While the prevalence of diabetes increases with age (Centers for Disease Control and Prevention [CDC], 2003), it is not clear that, in the absence of chromosomal disorders, congenital birth defects are associated with maternal age.

Hormone levels (e.g. cortisol): Fetuses exposed to glucocorticosteroids in the first trimester appear to have a lower median birth weight and are born at an earlier gestational age, which increases the risk for being underweight or having birth defects resulting from underdevelopment, but they have not been shown to exhibit an increased teratogenic risk (Gur, Diav-Citrin, Shechtman, Arnon, & Ornoy, 2004). However, they have been included within this discussion because of the known teratogenic effects of cortisol and other stress-related hormones in animal models (Goldman, Katsumata, Yaffe, & Gasser, 1997).

2.3.1.5   Covariates Related to Environmental Factors

Respiratory infections and febrile illnesses: Research has indicated that certain maternal infections and febrile illnesses, such as rubella and influenza, are related to birth defects (Jenkins et al., 2007). Mothers reporting any febrile illness during the first trimester of pregnancy have a two-fold higher risk of offspring with a heart defect (Ferencz, Correa-Villasenor, & Loffredo, 1997). However, there is little evidence to suggest that such infections are associated with IGT.

2.4   Data Sources for the Covariates

Data on the covariates to be used in this analysis will be obtained through various collection instruments. Data regarding a participating mother's family and personal history of birth defects, family and personal history of diabetes and/or obesity, race/ethnicity, SES, area of residence, age, smoking

status, specific medication use during critical time periods prior to and during pregnancy, use of nutritional supplements again during critical time periods, time of entry into prenatal care and physical activity levels will be collected through administration of several questionnaires. Data of biochemical origin, such as the mother's lipid profile and other cardiometabolic risk factors, serum inositol and other hormone levels, in addition to the mother's HgbA1c level, will be assessed from the first trimester blood sample. In addition, all genetic sequencing for targeted genes will also be analyzed. Data obtained from medical observation, such as parity of births, respiratory infections, febrile illnesses, sonogram results at the time of examination, will be recorded by a licensed medical practitioner. Medical record reviews will be performed to verify information obtained through the data collection efforts described above.

3.   Analytic Approach

3.1   Introduction

The National Children's Study is based on a representative sample of approximately 100,000 infants live-born to women residing in the United States. These births will be sampled in 105 geographic locations (see the Research Plan for sampling details). The pregnancy status of all eligible women of child-bearing age in these areas will be monitored for 4 years. All women living within the Study locations who become pregnant during the 4-year period will be enrolled in the study as early in pregnancy as possible in order to measure in utero exposures. Some women who state that they are seeking to become pregnant will in fact be enrolled in the NCS prior to becoming pregnant.

Although the NCS is a nationally representative cohort study of births, a nested case-control approach will be used for the analysis of the association between HgbA1c and birth defects. The nested case-control analysis approach offers two important advantages over an approach that analyses the full cohort. First, there is a significant advantage in terms of cost. Since the determination of a person's HgbA1c level costs approximately $22 per individual in 2007 in some research laboratories, it would be very costly to analyze HgbA1c levels for the full cohort. By restricting the analysis to all cases and a sample of a small number of controls per case, the cost of analyzing the blood samples is considerably reduced with only a modest loss of power for detecting effects.

The second important advantage of the case-control approach is that the repository samples of non-sampled controls remain available for use in other research. Given the number of probable analyses on the first trimester of pregnancy blood samples, this is a major consideration.

It should be noted that the number of infants with birth defects and other numbers used in the calculations below are initial estimates used only for illustrative purposes. They will need to be revised based on the results obtained during the actual data collection process.

3.2   Inclusion/Exclusion Criteria

Since the NCS is confined to live births for practical reasons of data collection, the Study will collect data only on the birth defect outcome status for those pregnancies that result in a live birth. The main analyses for this investigation of the association between HgbA1c levels and congenital birth defects will thus exclude miscarriages and stillbirths (but see below).

Excluded from this investigation will be births to mothers who experience Phenylketonuria (PKU). PKU, which is a rare autosomal recessive disorder of amino acid metabolism that affects 0.01 to 0.02 percent of births in North America, is unrelated to glucose metabolism. It is most often due to

deficiency of the enzyme phenylalanine hydroxylase which causes the accumulation of harmful metabolites, including phenylketones. If the mother is untreated, PKU can lead to several neurological birth defects (Luke & Keith, 1990).

Also to be excluded are births to mothers who during pregnancy used known teratogenic drugs or medications, such as Acutane, that are associated with induction of birth defects. These exclusions will be confined to births to mothers who used medications that are known to be causally associated with birth defects to the point that they outweigh any influence that glucose metabolism may have on congenital birth defects.

Chromosomal malformations such as trisomies, most notably Down syndrome, are not suspected as being influenced by glucose metabolism. As a result, those births that result in a diagnosis of Down syndrome, or any other trisomies, will be excluded from the analysis.

Another issue to be considered relates to women with pharmacologically treated diabetes. The plan is to retain the births to these mothers in the investigation. To guard against possible biases created by doing so, two analyses will be performed: one will analyze data from all women in the investigation while the second will exclude women with pharmacologically treated diabetes.

The findings from this study need to be interpreted in the light of the above exclusions. In particular, the number of miscarriages and stillbirths is sizable and many of the have malformations. Fetal mortality rates (20 weeks or later in gestation) are estimated at 6.4 per 1,000 (National Center for Health Statistics, 2003). Pauli and Resier (1994) reported that malformation syndromes and single malformations were causes of death in 78 percent of stillborns in a representative cohort. Malins (1978) reported that fatal malformations accounted for 50 percent of fetal losses in a cohort of diabetic pregnancies. While the magnitude of fetal death associated with fetal malformations is not known, the available data indicate it may be non-trivial. Since the main analysis will include only live births, a secondary analysis into the effects of first trimester HgbA1c levels on fetal death during the second or third trimesters will be conducted. This will also be a case-control study, with the cases being those mothers whose pregnancies result in fetal death and the controls being those controls used for the main analysis. It is estimated that there will be about 500-600 fetal deaths observed in the NCS sample of pregnant women. Fetal death is the main outcome variable for this analysis because it will be known for all the women involved. In addition, an investigation will be conducted to determine the number of the fetal deaths for which fetal death records can be obtained and the extent to which these records provide reliable information on the presence of congenital malformations. If reliable information on congenital malformations can be obtained for a large proportion of the fetal deaths, then another analysis that uses congenital malformations as the outcome variable will be conducted.

3.3   Selection of Cases

Within the context of this analysis, cases are defined as children with a medically diagnosed congenital birth defect identified during the first 24 months of life. Ideally, the study would take as the sample of cases all those infants who are diagnosed as having birth defects among all 100,000 mother-infant pairs in the full NCS sample. However, the sample size available for analysis is reduced for two reasons. First, some mothers in the NCS will not have provided the prenatal repository samples collected in the first trimester for use in measuring the HgbA1c exposure being studied in this analysis. Second, outcome data on birth defects will not be obtained for a small number of the live births due to withdrawal of consent, loss to follow-up, emigration, or death before the infant is 2 years old.

As discussed in Section 2.2, the exposure measurement required for this hypothesis is HgbA1c level as measured in the first trimester of pregnancy. Under the study design, HgbA1c levels will be assessed from a blood sample obtained from pregnant women during a first-trimester data collection visit. However, some women recruited into the NCS (approximately 10 percent) will not enter the study until after the first trimester of pregnancy and therefore their HgbA1c levels during the required period will not be known. The exposure measure will also be missing for a small percentage of women (approximately 4 percent) who refuse to provide a blood sample during the first trimester visit. As a result, it is estimated that first trimester HgbA1c levels will be available for approximately 86,400 women (i.e., 0.9x0.96x100,000).

While the presence of a birth defect may be diagnosed up to 24 months of age, in the majority of cases birth defects will be detected immediately after the infant is born. Outcome data will not be obtained for some of the births (approximately 3 percent) due to withdrawal of consent, loss to follow-up, or emigration, and for some infants who die in the first 2 years of life. For live births in these categories, questionnaire data, medical records, and/or death certificates will be used to determine if a congenital birth defect was diagnosed, and, if it was, they will be classified as cases. The others will be classified as having missing outcome data (recognizing that there is a high likelihood that they do not have birth defects due to the low prevalence of the identification of birth defects later in the 2 year period). The total number of live births for whom birth defects are not diagnosed before they leave the study is estimated to be 2,930 (i.e., 0.03x100,000 less an estimated 70 births for whom outcome data is obtained from other sources). Of these, 400 are estimated to be to mothers who lack a first trimester HgbA1c measurement.

The proposed analysis will thus be based on a sample selected from the approximately 83,870 mother-infant pairs for whom both exposure and outcome data are available. As discussed in Section 3.5.1 below, weighting adjustments will be applied to account for the possibility that mother-infant pairs with complete data may have different characteristics from those for whom HgbA1c levels and/or birth defects are unknown.

Based on an assumed prevalence rate for birth defects of 35/1,000 infants, the expected number of cases for use in the analysis is about 2,995.

3.4   Selection of Controls

For reasons of economy and the preservation of first-trimester blood samples, it is proposed to include only a sample of the approximately 80,875 infants without birth defects to serve as controls in the analysis. There are three primary issues to consider when selecting mother-infant pairs as controls for the analysis: what, if any, matching variables should be used; how many controls should be selected; and what sampling methods should be used. These issues are discussed in turn below.

3.4.1   Matching Cases to Controls

Matching in case-control studies ensures that the matching factors are equally distributed between cases and controls and thus removes any confounding effects of these factors. While controlling for confounders can be done in the sample design or during analysis, the former approach can be more statistically efficient. A critical requirement in the choice of any variables used to match in design is that the matching factors must be clearly and unambiguously causally prior to the exposure measure. If any doubt exists, it is better to assess the effects of the variable in the analysis, where it is possible to include it in some analyses and exclude it from others.

The initial proposal is to match on the primary sampling unit (PSU), i.e., geographic location, and time of pregnancy (e.g., year, season). The reason for choosing PSU is to accommodate the replication methodology that will be used for variance estimation (see Section 3.5.3). Matching on the year and/or season corresponding to the start of pregnancy may help to control for various temporal and/or environmental factors. The general approach proposed is to account for all other confounders in the analysis, unless there would be a marked loss in power through doing so. To identify potential confounders that need to be included in the matching process, the associations of the potential confounders with the outcomes will be computed. If any of these associations are so strong that handling the confounders in the analysis would likely lead to a substantial loss of power compared to matching in the design, consideration will be given to using these confounders as matching factors.

3.4.2　　　Number of Controls per Case

The greater the number of controls per case, the greater is the power for hypothesis testing and the greater is the precision of the estimates produced. The marginal increase in power in moving from three to four controls per case is 6.7 percent, from four to five is 4.2 percent; and the marginal increases diminish rapidly thereafter (Taylor, 1986; Ury, 1975). Also, the loss of precision and power from reducing the control sample size from that of the full cohort of possible controls with complete data (83,870) to the proposed sample size of four times as large as the case sample size (i.e., 14,970) is not that great while leading to substantial cost savings and preservation of blood samples. The compromise allocation of four controls per case is therefore chosen as the one that best balances costs against benefits.

3.4.3　　　Methods for Selecting Controls

First, cases and controls will separately be grouped according to their values on the matching factors, such as PSU, time of pregnancy, and any other variables chosen based on the analyses of their associations with the outcomes. It is unlikely that it will be necessary to control for more than one or two other variables by matching in design. Hence, there will be several cases in each group and a sample of matching controls that is four times the size of the case group will be selected. The controls will be selected using systematic probability proportional to size (PPS) sampling, where the size measure is the inverse of the nonresponse adjusted sampling weight. Selection of the sample in this manner results in a roughly equal probability of selection for each of the matched controls within a group.

This type of selection is known as "frequency matching," where a group of cases with similar characteristics is matched at a given ratio (1:4 in this instance) to a group of controls with the same characteristics. There are several operational and analytic advantages to frequency matching, and it is often used in survey settings (Korn & Graubard, 1999).

3.5　　　Weighting

3.5.1　　　Methodology and Rationale

The general approach adopted for the analysis is a "design-based approach" in which the estimates produced by the analysis are estimates for the U.S. population of inference, namely live births. The 100,000 live births in the full NCS constitute a national sample of live births. When this sample is appropriately weighted to compensate for unequal selection probabilities, nonresponse, and noncoverage,

the sample estimates are estimates for the nation.[1] However, a caveat, as discussed above, is that some of the sample mother-infant pairs lack data on the HgbA1c exposure measure and some lack information on the birth defect outcome measures. Steps need to be taken to compensate for these missing data in order to maintain the ability to produce national estimates.

There are a number of ways to handle the problem of the missing exposure and outcome data. One option would be to simply restrict the analysis to the mother-infant pairs for whom both exposure and outcome data are available, but selection biases may make the results unrepresentative of the U.S. population of inference. Another option would be to impute for missing data on HgbA1c levels and data on birth defects so that all infants in the NCS cohort could be used in the analysis. However, this approach would require a variance estimation method that accounts for the effects of imputation on the variances of the estimates produced, such as through the use of multiple imputations. Rather than employ the more complex imputation approach, weighting adjustments are proposed as the method for dealing with the missing exposure and outcome data. The full cohort weights of the approximately 83,870 mother-infant pairs with both exposure and outcome measures will be adjusted so that this sample is representative of the U.S. population of inference.

As discussed earlier, the full NCS sample will consist of 100,000 live-born infants, of whom approximately 86,400 are expected to have exposure measurements (HgbA1c) from their mothers' first trimesters of pregnancy. Among the 86,400 infants with exposure data, a certain number will be lost to the study before the 24-month period for assessing birth defects has elapsed. Some of these infants will have incomplete outcome data. It is possible to address both these missing data problems through carefully constructed weighting adjustments that take account of what is known about those with incomplete data (Brick & Kalton, 1996).

To compensate for missing outcome data from incomplete follow-up, adjustment factors will be calculated within selected weighting classes formed by exposure, demographic, and other variables related to missing data rates. The weighting classes would likely be formed based on length of follow-up, absence of evidence of birth defects during follow-up, and other factors such as mothers' HgbA1c levels being similar (if known) or unknown. The full NCS weights of infants with outcome data will be adjusted upwards within each weighting class to represent infants with missing outcome data in that class. For example, consider an infant who dies at age 6 months and for whom no information pertaining to birth defects is obtained from death certificates or other sources. Suppose also that the mother's first trimester HgbA1c levels are not known. This infant would be assigned to a weighting class where for all members of the class: mother's exposure is unknown; outcome data on birth defects is known; the infant lived for at least 6 months and had not been diagnosed with a birth defect up to that time (but may have been subsequently); and demographic characteristics such as socioeconomic status and mother's age are similar to those for the infant with missing outcome data. The sampling weight of the infant with missing exposure and outcome data would then be pro-rated across the members of the weighting class with missing exposure data but known status with respect to birth defects. These adjustments compensate for missing outcome data, leaving the issue of next compensating for missing exposure data.

Weighting adjustments will also be applied to compensate for infants with missing exposure data. This process need only include infants with known outcome data and their adjusted weights from the first step, since they now represent infants for whom birth defect status is unknown. Weighting classes will be formed using outcome values and other factors that are known to be related to maternal hemoglobin levels (e.g., socioeconomic status), and weighting adjustments will be computed in a manner similar to that described above.

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[1]See Chapter 8 of the NCS Research Plan for a discussion on the statistical weighting methods to be used for the NCS as a whole.

These weighting adjustments assume that data on exposure and outcomes are missing at random (MAR), i.e., that the probability of the data being missing is constant within the weighting classes (Little & Rubin, 2002). The adjustments compensate for unequal probabilities of the data being missing across weighting classes, and they will reduce bias to the extent that these probabilities are unequal and the weighting classes are associated with missing data.

3.5.2   Weighting in Case-Control Studies

In most nested case-control studies, the controls are sampled at much lower rates than the cases and also different groups of controls are sampled at widely differential rates in order to make the control distribution across the groups correspond to that of the cases. In this situation, the use of standard survey weights based on the general population as the reference population (i.e., the population of inference) will lead to large variability in estimates that compare cases and controls. An alternative approach is to use the distribution of characteristics among the case population as the reference distribution. This approach is consistent with that used in most case-control studies and will be adopted here: the reference population is all cases in the U.S. population of infants. The weights described above for the cases were developed for this purpose. The weights for the control sample will be similarly developed to represent the characteristics of the case population. In this approach, the weights of controls in a group are scaled to the sum of weights of the cases in that group, thus making the reference population the case population, not the total population (DiGaetano & Rizzo, 2003).

While the use of this weighting method in the planned analysis will result in some loss of precision, the approach is preferable to an unweighted analysis of a self-selected sample, the results of which cannot safely be generalized. Effects of elevated maternal glucose levels obtained from the proposed weighted analysis will represent the average exposure effect on the national population of cases or on any specific subset of that population.

3.5.3   Replicate Weights

The NCS is based on a complex sample design involving stratification and clustering by PSUs and by segments within PSUs. One way to reflect the variance due to this type of sample design is to use replication variance estimation methods. This methodology requires the development of a set of replicate weights in addition to the set of final weights described in Sections 3.5.1 and 3.5.2.

Replicates are subsets of the sample formed so that each replicate is a representative sample of the entire study population. Typically, replicates are formed by dropping one or more PSUs out in turn. However, it needs to be noted that large PSUs that are selected with certainty are in fact strata, not PSUs. It is the sub-units within these strata that are the real PSUs, and it is these sub-units that are dropped out sequentially to form replicates. Replicate weights are computed separately for each subset so that the weighted replicate estimates represent the study population. Sample statistics (e.g., means, proportions, regression coefficients) are computed using the full sample weights and then separately for each replicate using the replicate weights. The variability in the replicate results provides the basis for estimating the precision of the estimates produced in the analysis, in a manner that reflects the sample design and the weighting process.

3.6   Imputation of Missing Data for Covariates

As explained in Section 3.5.1, missing data on HgbA1c levels and/or birth defects will be handled by weighting adjustments. In addition, covariate information may be missing due to item nonresponse. The percentage of missing data for each analysis variable will be evaluated and appropriate procedures will be used to impute for missing values. The procedure used for each variable will depend on the extent and type of missing data but possible methods include hot deck imputation, semiparametric imputation, or logical imputation based on related variables.

3.7   Power Calculations

We assume that approximately 14,970 infants will be included in this analysis. In the analyses, it is anticipated that the level of HgbA1c will be treated as a continuous variable (likely with curvilinear terms or a spline in the logistic model). Unfortunately, data on HgbA1c levels for women in the first trimester of pregnancy, the primary exposure measure, are not readily available. The power calculations have therefore been carried out by treating HgbA1c as a binary variable. The calculations use a value of 4 percent in the "high" group, as would apply if only the very high levels of HgbA1c were associated with birth defects. The power calculations presented here are therefore likely to be conservative. The table below provides estimates of power for detecting odds ratios in the range of 1.50 to 3.00 under these assumptions and also under an assumption of a design effect for odds ratios of approximately 1.30 based on the expected number of cases per PSU and estimated intra-class correlation for the NCS for birth defects.

Outcome	Rate per 1,000 births	Expected sample size	Odds ratio				
			1.50	1.75	2.00	2.50	3.00
Heart defects	6	510	0.31	0.54	0.74	0.94	0.99
All major birth defects	35	2,995	0.91	>0.99	>0.99	>0.99	>0.99

The results in this table demonstrated that this investigation has very high power for detecting clinically significant associations between elevated HgbA1c levels and all major birth defects as a group. Moreover, the analyses will also be able to support stratified analyses of subgroups of the sample in order to investigate potential effect modifiers, an important aspect of the analyses.

The statistical power to detect the effects of impaired glucose tolerance on heart defects is lower but still adequate for odds ratios greater than about 2. It may also be possible to examine as outcomes other groupings of birth defects that are suggested by the literature to be associated with gestational hyperglycemia.

4.   Analysis Procedures

4.1   Overview

The data analysis will be conducted in several steps. First, data must be combined from various files to create a data file for analyzing the associations of potential confounders with birth defect outcomes (all birth defects and heart defects only). The results of these analyses will be used to guide the choice of confounders to be used in the matching. The sample of matched controls will then be selected and an analytic data file that includes all the variables to be used in the analyses will be created. Next, preliminary frequencies and descriptive statistics will be generated for all analytic variables. A

preliminary set of analyses will examine bivariate relationships between covariates and exposure, covariates and outcomes, and exposure and outcomes. The main analysis and final step of the process will involve fitting a conditional logistic regression models for the presence of birth defects given HgbA1c levels and other covariates.

4.2   Preliminary Frequencies and Descriptive Statistics

Once an analysis data set has been produced, unweighted frequencies and descriptive statistics for all key variables will be reviewed. The main purpose of this step will be to check for correct coding of the data, logical relationships between related variables, the need to collapse categorical variables, extreme values, skewed, and other distributional features that might affect data analysis, and to assess the extent of missing data.

As a result of this step, some corrections or recoding of the data may be required. Once these issues have been addressed, the changes will be verified and additional output will be reviewed as necessary.

4.3   Bivariate Relationships

The purpose of this step is to gain insight into the pairwise relationships between covariates and HgbA1c levels, between covariates and congenital birth defect outcomes (both overall and for heart defects alone), and between HgbA1c levels and birth defects. First, two-way cross tabulations will be generated for each pair of variables by categorizing continuous and many-leveled ordinal variables, as necessary. Where outcomes are involved, separate tables will be run for all birth defects and for heart defects. These frequencies will be run with and without sampling weights. Tests of association between variables will be conducted on the weighted tables using appropriate software to account for the complex sample design. These results will be reviewed by the analysis team and used to inform the model building process in the final step.

4.4   Conditional Logistic Regression Analyses

Conditional logistic regression is the primary tool for the analysis of data from matched case-control studies. It is used to assess the effects of covariates on the relative odds of a particular outcome and, in particular, to remove the influence of confounders from the estimate of effect due to exposure. The form of the regression model is

$$\log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \alpha_i + \mathbf{x}'_{ij}\boldsymbol{\beta}$$

where, in the context of this analysis, π_{ij} is the probability that the jth infant in the ith matched group is diagnosed with a birth defect in the first 24 months of life, and $\mathbf{x}_{ij}$ is a vector of covariates that includes the status of the jth infant's mother with respect to HgbA1c level in the first trimester of pregnancy, as well as all confounders. The covariate vector can include both individual-level characteristics and characteristics that are shared by all infants in the matched case-control group.

The conditional logistic regression model allows each matched group of infants to have a unique risk of birth defects by a group-specific intercept α_i, and then assumes that the covariates have a common effect on the odds of birth defects over all matched groups represented by the coefficient vector $\boldsymbol{\beta}'$.

To perform this analysis, the conditional logistic model can be fit using software that fits the discrete proportional hazards model for tied event times since both models have the same likelihood function. In this approach, the matched groups are treated as strata, case status is treated as the censoring variable (1 for cases, 0 for controls), and a dummy time variable is created (e.g., 1 for cases, 2 for controls)(see, for example, Lachin, 2000.)

Identification of an appropriate model will initially be facilitated by using a stepwise selection procedure to determine whether a covariate should enter and remain in the model. However, covariates identified by subject-matter experts as being important factors in birth defects, glucose functioning, or general health may be forced into the model. Likelihood or deviance measures will be used to assess overall model fit and residuals will be examined to address model diagnostics. Once a set of main effects has been identified, potential interactions will be tested for significance. A separate model will be fit for each of the two outcome variables. Potential effect modifiers will be investigated either by including in the model an interaction term between the effect modifier and the exposure measure, or by stratifying the model to see if the effect of exposure differs across strata defined by levels of the potential effect modifier.

These models will be estimated using the sampling weights described in Section 3.5.2, and standard errors and tests of significance will be computed based on replicate methods using software such as WesVar or SUDAAN (Westat, 2007; Research Triangle Institute, 2004). Team members will then review and interpret the results of the analyses, such as model fit statistics, estimated regression coefficients and odds ratios for each independent variable, and significance tests.

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