Prenatal Genetic Diagnosis by Genomic Sequencing: A Prospective Evaluation (PrenatalSEQ)

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1 Introduction

1.1 Study Abstract

Whole exome and whole genome sequencing (WGS) have expanded the ability to determine the genetic etiology of previously undiagnosed disorders. This study is a multicenter prospective cohort study to evaluate the emerging technology of sequencing for the management of fetuses with structural anomalies. The hypothesis is that a significant subset of fetal structural anomalies has a genetic etiology identifiable by sequencing and that prenatal knowledge of this information will improve perinatal care, reduce unnecessary diagnostic testing, reduce the cost of care, and improve quality of life for both the child and the family. The aims of this study are to investigate these multiple aspects of prenatal sequencing in a single study with an innovative integrated prospective design, which will permit a robust evaluation of the benefits and risks of delivering diagnostic and prognostic genetic testing results in a prenatal setting.

The study will determine, in a sequential population of pregnancies with selected fetal structural anomalies and a negative or non-causal chromosomal microarray (CMA), the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing. To determine the impact of this information on clinical care, a control population of unsequenced pregnancies with similar structural anomalies will be prospectively recruited and the infants from both cohorts will be followed up to 1 year of age. This study component will evaluate differences in healthcare management and cost through discharge from hospital post-delivery, and perinatal and infant outcomes through 1 year of life. The educational, counseling and psychosocial impact of sequencing results during the prenatal period, in the nursery and through 1 year of life also will be evaluated. Since the analytical and clinical tools needed for the full translation of sequencing into care are still developing, optimization of bioinformatic tools to improve identification of pathogenic and likely pathogenic mutations associated with prenatal phenotypes of established disease genes will be investigated, as well as identification of new genes associated with presently undiagnosed fetal/neonatal phenotypes. This study will provide an in-depth evaluation of the prenatal diagnostic value of sequencing prior to its responsible introduction into practice and will provide independent data to guide its translation.

1.2 Objectives

The main objective of this multi-center collaborative study is to evaluate sequencing (both whole exome sequencing [WES] and WGS) as a prenatal diagnostic tool in pregnancies with a structural anomaly and a negative or only non-causal karyotype/ chromosome microarray analysis (CMA). Specifically, the aims are as follows:

1. To determine in pregnancies with structural anomalies which are CMA negative (or have only non-causal findings), the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing.
    a. Examine the relative yield of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by WGS compared with analysis of the coding sequence alone (simulating WES).
2. To evaluate the impact of having sequencing results available prenatally on clinical management, including health care utilization and costs, comparing outcomes of pregnancies and neonates with *in utero* diagnosed structural anomalies that have undergone prenatal sequencing with those that have not.
3. To evaluate the educational/counseling and psychosocial needs of pregnant couples having a fetus with a structural anomaly and assessing the psychological impact of sequencing and return of results on couples and determine the correlation with demographic factors, knowledge and attitudes about genetics, baseline parental attitudes and beliefs, and genetic test results.

4. To expand the diagnostic yield and clinical utility of fetal sequencing by developing and optimizing bioinformatic tools capable of efficiently and rapidly identifying pathogenic and likely pathogenic variants with prenatal presentations.

1.3 Purpose of the Study Protocol

This protocol describes the background, design and organization of the study and may be viewed as a written agreement among the study investigators. It is approved by the funding agency (NICHD), the study Steering Committee, the Data Safety Monitoring Board (DSMB), the single Institutional Review Board (IRB) of the Clinical Coordinating Center and participating laboratories before recruitment begins. Any changes to the protocol during the study period require the approval of the Steering Committee and the IRB.

A manual of operations supplements the protocol with detailed specifications of the study procedures.

2 Background

2.1 Introduction

With advances in prenatal imaging, increasingly there is the ability to detect birth defects prenatally and use this information to optimize perinatal and neonatal management. Simultaneously, molecular genetic diagnostics have facilitated more precise identification of the underlying cause of these birth defects and provided additional prognostic information to improve prenatal and postnatal management. The first major improvement in prenatal genomic diagnosis was chromosome microarray analysis (CMA) which identifies copy number variants (CNVs) of 100 kb to 10Mb that are below the resolution of most karyotypes. A multicenter study evaluating the clinical utility of prenatal CMA reported that CMA identified the same aneuploidies and unbalanced rearrangements as karyotyping but also identified additional, clinically significant cytogenetic information.[1] Among women with a normal karyotype, CMA identified clinically relevant deletions or duplications in 6.0% with a fetal structural anomaly and in 1.7% of those with indications of advanced maternal age or positive aneuploidy screening results. The frequency of CNVs varied by anomaly, with the highest frequency (16%) occurring in fetuses with cardiac defects.

Congenital anomalies affect 2-4% of all infants and are responsible for 20% of perinatal deaths.[2] Prenatal genetic evaluation of fetal anomalies is limited to karyotype and CMA performed on amniocytes or chorionic villi. The karyotype is abnormal in 20-25% and CMA is abnormal in 5-10% of fetal anomalies, leaving 60-70% without a genetic diagnosis.[3] WGS is the process of determining the complete DNA sequence of an organism's genome at a single time and includes both the coding and non-coding regions. Whole exome sequencing (WES) analyzes only the coding regions of the genome (exons; DNA code for making proteins). Until recently, researchers have elected to use WES because it costs less and because the functional consequences of positive findings occurring within coding regions of genes are much easier to interpret. Because of its high cost and incomplete knowledge about the role of non-coding variants in disease, WGS has primarily been used as a research tool. This may change as sequencing costs decrease and knowledge of the functional consequences of non-coding genetic variants increases. Numerous recent reports describe the speed and reliability of WGS in the context of rapid clinical sequencing since library preparation is not required[4-6] making WGS an advantageous technology for rapid genomic testing in the future.

Prenatal sequencing has the potential to alter the life course and reduce morbidity and mortality by guiding pregnancy management, delivery plans and/or the treatment of fetuses and newborns with some genetic abnormalities.[7] One example is *in utero* fetal intervention now performed for certain anomalies including aortic stenosis to avoid hypoplastic left heart syndrome, urinary diversion for posterior urethral valves, drainage of pleural effusions and chylothorax, surgical repair for spina bifida, tracheal occlusion for congenital diaphragmatic hernia, and amnio-infusion for renal agenesis.[8-14] For all of these, knowing their genetic etiology aids clinical management decisions by providing information about other associated medical features that affect the likelihood of success of the intervention. In addition, prenatal diagnosis of some metabolic conditions could guide early postnatal treatment and avoid metabolic crises that can lead to long term neurocognitive deficits. For example, pyruvate dehydrogenase deficiency can present prenatally with structural anomalies and is modifiable by a ketogenic diet and thiamine supplementation.[15,16] Finally, mesenchymal stem cell transplantation for osteogenesis imperfecta has been reported to increase skeletal mineralization and growth velocity without adverse outcomes.[17-20] With developments in gene and stem cell therapy, this type of treatment may increasingly become an option for other conditions.

2.2 Preliminary Studies

2.2.1 Postnatal Studies

WES, which analyzes the coding regions of the genome (exons) is a powerful diagnostic tool in adults and children with genetically heterogeneous conditions.[21,22] Compared with a 10% diagnostic yield with karyotype and CMA, postnatal WES has diagnostic yields of >30% in adults and children with phenotypes suggestive of monogenic disorders.[22] The mean and median overall diagnostic yields of a series of 16 studies was 36% and 38%, respectively, but was highly dependent on the *a priori* risk for a monogenic disorder. In addition to the diagnostic utility of WES in these populations, WES analysis has resulted in discovery of new disease genes with pleiotropic phenotypes.

WGS is now gaining traction as a diagnostic and discovery strategy for adults and children with a suspected genetic disorder that remains undiagnosed after WES analysis, or as a first-line approach in lieu of WES. WES includes an exon capture step, which adds time and reagent costs, and biases against coverage in GC-rich regions. WGS does not include this selection step, providing more uniform coverage which allows a lower mean read depth, offers ability to detect copy number variants (CNVs) with higher resolution than CMA and more complex balanced re-arrangements. The lower read depth however will also make it more difficult to detect mosaic mutations which are an important source of mutations in neurodevelopmental disorders including autism and epilepsy.[23,24] WGS was found to detect up to 3% of protein-coding variants missed by WES.[25] Recent WGS studies have found more causative variants in coding and non-coding regions in autism.[6] In addition, an increased burden of rare *de novo* variants in non-coding regions for congenital heart disease and diaphragmatic hernia has been seen (Chung, WC; unpublished data).

2.2.2 Prenatal Studies

Only a few case series of prenatal WES have been published to date with a wide range of indications, including pregnancy terminations, fetal demises with fetal anomalies, euploid fetuses with sonographically detected single or multiple fetal structural anomalies, and increased nuchal translucency (NT) > 3.5 mm have been reported[26,27] with widely variable diagnostic yields of 6.2 - 80%. The 2 largest series of trio exomes on over 200 fetal anomalies each had overall diagnostic rates of 6.2 and 7.5%, but was higher (14.3% and 16.0%) for fetuses with multiple anomalies.[27]

Table 1. This table describes the frequency of pathogenic variants by whole exome sequencing in various publications

First Author, Year	# Cases	Cohort Criteria	Method	Pathogenic Variants*
Normand, 2018[28]	146	Fetuses with ultrasound anomalies and a suspected Mendelian disorder	62 Trio	46/146 (32%)
Aarabi, 2018[29]	20	1 or more major structural congenital anomaly detected by ultrasound	Trio	4/20 (20%)
Fu, 2017[30]	196	Fetuses with structural abnormalities	147 Proband-only 49 Trio	34/147 (23.1%) 13/49 (26.5%)
Lei, 2017[31]	30	Fetuses with congenital anomalies of the kidney and urinary tract	23 Proband-only	3/23 (13%)
			7 Trio	1/7 (14%)

			Total	4/30 (13.3%)
Vora, 2017[32]	15	Fetuses with multiple congenital anomalies highly suggestive of an underlying genetic disorder	Trio	7/15 (47%)
Yates, 2017[33]	84	Fetuses with ultrasound abnormalities that resulted in fetal demise or pregnancy termination	29 Proband-only	4/29 (14%)
			45 Trios	11/45 (24%)
			6 Quads/4 Maternal Duos	2/10 (20%)
			Total:	17/84 (20%)
Pangalos, 2016[34]	14	Prenatal ultrasound abnormalities or malformations	Proband-only	6/14 (43%)
Alamillo, 2015[35]	7	Multiple congenital anomalies on prenatal ultrasound	Trio	4/7 (57%)
Drury, 2015[36]	24	Fetuses with an increased NT (>3.5 mm) or other ultrasound abnormality	14 Proband-only	2/14 (14%)
			10 Trio	3/10 (30%)
			Total	5/24 (21%)
Carss, 2014[37]	30	Structural abnormalities identified on prenatal ultrasound	Trio	3/30 (10%)
Yang, 2014[38]	11	Terminated fetuses with anomalies	Trio	6/11 (54%)

2.2.3 Healthcare Utilization and Cost

Integration of WES or WGS into clinical care requires not only demonstration of the ability to identify the underlying etiology of a disorder but also requires evidence that care is improved, providing value to the health care system. While WGS is currently a relatively expensive test (≥$15,000 per trio), the information may lead to significant alterations in care utilization. Given that healthcare costs can be $250,000 or more in the first year of life for some complex congenital anomalies, the genetic diagnostic information may be net cost saving. Even if not cost-saving, if clinical outcomes are improved significantly, the incremental cost of WGS may be justifiable based on its cost effectiveness. Recent studies in critically ill neonates and infants have suggested that establishing a diagnosis leads to more focused management and reduction in healthcare utilization and reduced cost. Meng et al evaluated diagnostic WES for 278 critically ill infants within the first 100 days of life and found a genetic diagnosis in 36.7%.[39] These new diagnoses led to care modification in 52%, including initiation of new subspecialist care, redirection of care, changes in medication/diet, or completion of major procedures (e.g., transplant). The greatest impact was in infants receiving rapid "critical trio sequencing".[39] A meta-analysis found that change in clinical management by WGS results was 27% (4 studies with 136 children) compared with 17% by WES (12 studies and 992 children) and 6% by CMA (8 studies of 4,271 children).[40] Another study evaluating the cost-effectiveness of WES in a pediatric setting reported the mean duration of the diagnostic odyssey was 6 years, and that the diagnostic trajectory for WES performed at the initial tertiary presentation resulted in an incremental saving of $6800 per additional diagnosis compared with the standard diagnostic pathway.[41] In summary, studies have suggested that sequencing early in the disease course may provide the maximal benefit by modifying clinical management, reducing the time to diagnosis and cost.

2.2.4 Educational & Psychosocial Needs

The added prognostic information about health, treatability, life expectancy and neurodevelopmental, behavioral and cognitive function is far beyond what is learned from ultrasound findings and standard testing, and can have a powerful impact on parents. Identification of fetal genomic information has significant value to the management of the pregnancy. Ultrasound offers an anatomic phenotype but is incapable of evaluating long-term neurocognitive potential, limiting accurate prognostication and counseling. Karyotyping and CMA can offer disease specific counseling in a combined ~30-40% of detectable cases to provide couples with the information necessary to make informed reproductive decisions, leaving significant ambiguity in 60-70%. Equally important, knowledge of the genotype can direct additional fetal, neonatal and pediatric management.

Efforts to understand the psychosocial and behavioral impact of integrating genomic technologies into adult and pediatric practice are ongoing[42-44], but to date, little empirical work has been done to understand the unique challenges of applying genomic sequencing to a prenatal population.[45] Attitudes towards prenatal screening and diagnosis are influenced by ethnicity, socioeconomic status, cultural and religious beliefs, and experiences with disability.[45-49]

While WGS results may illuminate the situation when a well-known genetic condition is diagnosed, there are challenges in counseling for some of the newer genetic conditions for which less data are available. In CMA, variants of uncertain clinical significance (VUS) and those associated with variable expressivity occur in a proportion of cases with structural anomalies (5.3%) and require extensive genetic counseling.[50] Other counseling issues include the identification of adult onset disorders of both the fetus and parent. To understand attitudes and unmet needs in the adoption of CMA testing semi-structured interviews with parents and counselors were performed to evaluate pre-test counseling, reporting of results, and assessing patient and provider experiences.[47] From the patient perspective, 5 key themes were identified 1) accepting CMA testing was an easy decision to make as patients would obtain additional information on their baby's health at no additional cost, 2) patients were blindsided by the results in cases where they initially received a normal karyotype result followed by an abnormal CMA, 3) abnormal results left patients shocked, anxious, confused and overwhelmed, 4) patients needed support to manage, understand and act on the microarray results, and 5) uncertain findings were felt to be toxic knowledge and patients wished they did not receive the results. It is anticipated that the needs for prenatal sequencing will be similar to those of prenatal CMA and that prior studies will inform the educational and support materials developed for prenatal sequencing.

2.3 Rationale for the Study

Sequencing itself is a transformative technology to identify comprehensively genetic variants accounting for a phenotype, but its application to prenatal diagnosis has not been investigated prospectively in a large cohort.[22] This study population of unselected consecutive cases will be unique in that the majority of published series to date have only included select phenotypes felt to have a genetic etiology. It is possible that these studies have overestimated the frequency and underestimated the phenotypic variability of *in utero* genetic disease based upon the case ascertainment. A more comprehensive, less biased evaluation of the molecular etiology of birth defects should lead to the discovery of previously unknown genes and phenotypic associations. To date, only descriptive series of the diagnostic yield of selected prenatal WES or WGS have been reported on fetuses with a structural anomaly, but none have evaluated the benefit of a specific genetic diagnosis.[36,37,51-53]

This study will be the first to perform a cost-effectiveness analysis to evaluate whether prenatal knowledge of the genetic cause of a fetal single-gene disorder will lead to altered fetal/neonatal management, costs, and outcomes. Also important are the pertinent psychosocial effects of introducing sequencing into a prenatal setting.

Before integration into standard care, the diagnostic capabilities of prenatal WGS must mature by developing new bioinformatic tools to specifically facilitate accurate and efficient prenatal interpretation and novel gene discovery. This can be accomplished initially by analyzing and reporting the variants from the coding regions of the genome and then subsequently analyzing the non-coding regions, allowing comparison of the differences in diagnostic yield. The current missing pieces are insufficient WGS data in fetuses with abnormalities, the lack of variant analysis algorithms tuned to the prenatal setting, and insufficient publicly deposited data to inform clinical care for prenatal genomic testing and rare developmental disorders. As more data become available (WGS data from adults, more WES/WGS from more diverse communities, Human Cell Atlas data, and ATAC seq data from appropriate times in development), it will increasingly be possible to interpret the coding and noncoding regions that are relevant to developmental disorders.

3 Study Design

3.1 Design Summary

This multicenter, prospective observational cohort study will evaluate prenatal sequencing among pregnancies with fetal structural anomalies recruited at three university based medical centers and evaluated at three university genetic laboratories. A total of 1,100 pregnancies with fetal structural anomalies and meeting eligibility criteria will be enrolled into the study. Of these, 750 will undergo prenatal genomic sequencing (prenatal sequencing group) and the remaining 350 pregnancies will not have any prenatal genomic sequencing (unsequenced prenatal group). Enrollment of pregnancies with an isolated nuchal translucency measurements ≥ 3.5 mm will be restricted to 5% within each group (sequenced and unsequenced) and isolated estimated fetal weight <5th %ile also will be restricted to 5% for each group (sequenced and unsequenced).

The prenatal sequencing group will be used to determine the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing and the relative yield of sequencing. The prenatal sequencing group will be compared with the unsequenced prenatal group to evaluate health care management, health care utilization and cost, perinatal outcomes, and the psychosocial needs of pregnant couples. Mothers, fathers and infants will be followed through 1year postpartum.

3.2 Eligibility Criteria

3.2.1 Prenatal sequencing group

3.2.1.1 Inclusion Criteria

1. Fetus identified by ultrasound and/or MRI with at least one of the following:
    a. One or more major structural anomalies (Appendix A)
    b. A nuchal translucency measurement of ≥ 3.5 mm
    c. A fetus less than 24 weeks 0 days gestation with normal anatomy and sonographically estimated fetal weight <5th %ile without maternal hypertension, type I diabetes, or other maternal disorders known to alter fetal growth.
2. Negative prenatal CMA (or those with CMA findings not related to the ultrasound finding)
3. Singleton gestation
4. Gestational age less than 36 weeks, 0 days to allow for availability of sequencing results before delivery

3.2.1.2 Exclusion Criteria

1. Maternal or paternal age less than 18 years old
2. Proven infectious or teratogenic cause of fetal anomaly
3. Planned termination of the pregnancy
4. Unavailable blood or saliva samples from both biologic parents prior to sequencing
5. Parental unwillingness to participate in 1 year postnatal follow-up
6. Language barrier (non-English or Spanish speaking)

7. Delivery planned at a site other than one of the study centers or associated hospitals
8. Previous consent to the unsequenced prenatal group or enrollment in a previous pregnancy

3.2.2 Unsequenced prenatal group

3.2.2.1 Inclusion Criteria

1. Fetus identified by ultrasound and/or MRI with at least one of the following:
    a. One or more major structural anomalies (Appendix A)
    b. A nuchal translucency measurement of ≥ 3.5 mm
    c. A fetus less than 24 weeks 0 days gestation with normal anatomy and sonographically estimated fetal weight <5th %ile without maternal hypertension, type I diabetes, or other maternal disorders known to alter fetal growth
2. Negative prenatal or postnatal CMA (or those with CMA findings not related to the ultrasound finding)
3. Declined prenatal sequencing
4. Singleton gestation

3.2.2.2 Exclusion Criteria

1. Maternal or paternal age less than 18 years old
2. Proven infectious or teratogenic cause of fetal anomaly
3. Positive prenatal NIPT screening for trisomy 21,18 or 13. Positive 22q11.2 prenatal NIPT testing with consistent ultrasound findings is also an exclusion.
4. Planned termination of the pregnancy
5. Parental unwillingness to participate in 1 year postnatal follow-up
6. Language barrier (non-English or Spanish speaking)
7. Delivery planned at a site other than one of the study centers or associated hospitals

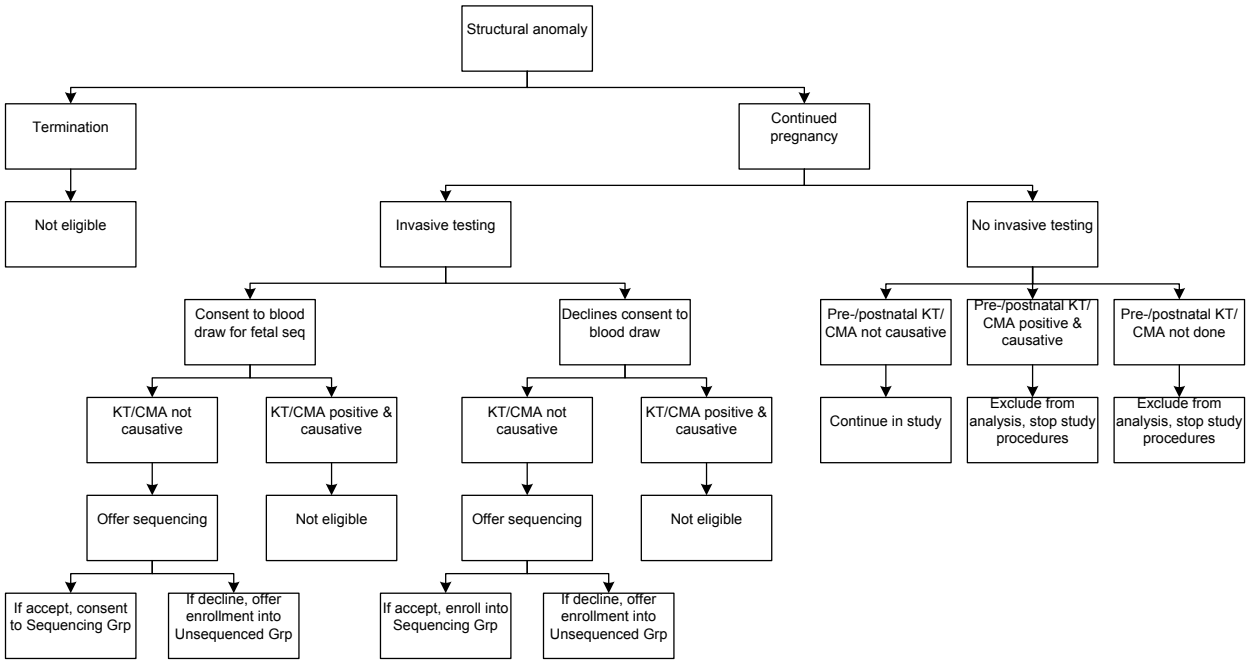
4 Study Procedures

4.1 Screening for Eligibility and Consent

Eligible patients with specified fetal structural anomalies will be identified by prenatal ultrasound and/or fetal MRI. Center investigators and their staff in the outpatient ultrasound prenatal diagnostic units will be responsible for identifying eligible patients. To maximize recruitment, coordinators at each center will screen the prenatal diagnostic logs on a daily basis to identify eligible patients.

Upon recognition of a specified fetal anomaly, the maternal fetal medicine physician (MFM)/ ultrasonographer and/or a genetic counselor will explain the ultrasound finding as per routine clinical care. At the conclusion of the initial evaluation/consult, women will be informed by the physician/counselor and/or coordinator about the study. Once the potential participant indicates willingness to hear more about the study, a study coordinator will meet with the parents, review eligibility, and explain the study. Women who choose to undergo prenatal invasive testing will be approached for the prenatal sequencing group but will not be enrolled until a negative or non-causal karyotype/CMA is confirmed (with the exception of those enrolled into expedited prenatal sequencing, section 4.2.1). Women who choose not to have any diagnostic testing or decline sequencing will be approached for participation in the unsequenced prenatal group. All women with a specified fetal anomaly will be recorded on a screening log that collects screening ID, date screened, planned invasive prenatal testing, ultrasound anomaly, screening status code and reason for study ineligibility (if applicable). A participant flowchart is shown in Figure 1.

Figure 1. Screening flowchart



4.1.1 Informed consent

Written informed consent must be obtained from participants and the biological father of the fetus prior to any study procedures. Three consents may be employed for this study:

- Short consent for potentially eligible women in the prenatal sequencing group that allows for collection of study samples prior to determination of the CMA and karyotype result. If CMA/karyotype results come back negative, eligible participants will sign the full study informed consent form to proceed with sequencing.
- Full study consent for the prenatal sequencing group that describes the specific group's participation in the study and risks
- Full study consent for the prenatal unsequenced group that describes the specific group's participation in the study and risks

Participants in both the sequenced and unsequenced groups will be assured that their participation in the study is purely voluntary and that patient care will not be affected if they decline participation in the study. Patients who are not fluent in English will be enrolled by a person fluent in Spanish. Both verbal and written informed consent and authorization will be obtained in that language; if this is not possible the patient will be excluded.

4.2 Enrollment

4.2.1 Prenatal sequencing group

For the prenatal sequencing group, chorionic villus sampling (CVS) or amniocentesis will be performed per standard of care. For CVS sampling, a portion of the villi, cultured villi or extracted DNA will be sent to the sequencing laboratory. For amniocentesis, the usual draw of 40cc of fluid will be divided by the cytogenetics lab, with a portion used for clinical testing (minimum standard amount as per diagnostic laboratory practice) and from the remainder, DNA will be extracted for sequencing in a CLIA laboratory. Additional DNA for sequencing will be extracted from the cultured cells and the sequencing lab will determine which is the optimal source for sequencing, after quality and quantity verification.

Potentially eligible participants will sign the screening consent form prior to collection of parental samples. Thirty ml of maternal blood will be collected for sequencing and future research. Paternal blood (up to 10 ml) or buccal swab/saliva will be collected for sequencing and future research. Every effort will be made to obtain samples from both parents prior to or at the time of the diagnostic procedure. If the father is absent, a sample will be obtained as soon as possible. If no paternal sample is obtained, the participant will be classified as a screen failure and will not be enrolled into the study. The parental samples will be sent for DNA extraction as soon as they become available.

If the clinical karyotype, CMA or any other non-sequencing test result is known to be positive prior to sequencing, and felt to be causative of the anomaly, the karyotype/CMA results will be recorded, the participant will be classified as a screen failure and not enrolled, and parental samples/DNA will be discarded.

If the clinical karyotype/CMA results are negative (or non-causative), eligibility criteria will be confirmed, the participant and biological father will sign the study consent form either in person or electronically, parental samples will be collected if needed, and genome sequencing will proceed. Parents declining participation at this time will be considered as having refused consent; these participants may be offered participation in the unsequenced prenatal group if eligibility criteria are met. Those choosing to terminate their pregnancy based on ultrasound/CMA findings are ineligible.

"Expedited" prenatal sequencing

An alternative to the prenatal diagnostic flow described above permits prenatal sequencing to be performed in parallel with the CMA, i.e. before the fetus is confirmed as CMA negative. This diagnostic workflow is at the discretion of the study research team and participants will be considered enrolled once they have signed the study consent and parental samples have been collected. If the CMA is negative or

unrelated to the fetal anomaly, the case will continue to be part of the prenatal sequencing group and included in the analyses. In pregnancies in which the CMA result is found to be positive (and presumed causal of the anomaly), sequencing will be discontinued or if already completed the results will be recorded and reported to the family in the appropriate manner. The karyotype/CMA results will be recorded; however, follow-up of the pregnancy through delivery will not take place, these participants will not be included in the analyses and not contribute to the sample size.

4.2.2 Unsequenced prenatal group

The unsequenced prenatal group will include participants that decline invasive testing and those that plan to have invasive testing but decline sequencing (Figure 1). For participants that plan to have invasive testing but decline sequencing, enrollment into the study will occur after the clinical karyotype/CMA results are confirmed negative and the participant signs the prenatal unsequenced consent. Participants that decline invasive testing will be enrolled at the time of diagnosis of the fetal anomaly and they sign the unsequenced prenatal group consent. Women from this group who later elect invasive testing are not eligible to enroll in the prenatal sequencing group but will be included in the unsequenced prenatal analyses if the karyotype and CMA are confirmed negative or non-causal. It is anticipated that most patients that do not have invasive testing typically will have CMA performed after birth as part of clinical care. Continued follow-up of the pregnancy and 1-year postpartum period will not take place if a positive causal CMA is identified.

Postpartum genomic sequencing may be performed at the discretion of the clinical care team as part of routine care.

4.3 Baseline Data Collection

In addition to data collected for eligibility and consent, the following information will be obtained at enrollment from either medical record review or participant interview:

- Maternal and paternal: age, race, ethnicity, education, income, household composition, religion, religiosity, number of children, and family history
- Maternal medical and psychiatric history including maternal history of disorders with teratogenic risk (e.g. diabetes, teratogen exposure including medications, diseases during pregnancy (e.g. CMV, Zika)
- Obstetrical history including
    - Clinical estimated date of delivery and type of conception
    - Pre-procedure ultrasounds including date and detailed information on anomaly(ies)
    - Number of prior pregnancies and gestational age of delivery
    - Unexplained infertility
- Family history of learning disabilities, genetic disorders, congenital anomalies, stillbirths or infant deaths, significant medical problems, fetal/child structural or growth findings, consanguinity, deafness and psychiatric illnesses.
- Prenatal screening and diagnostic test results; carrier screening and results; blood type and screen; rubella immunity status

A copy of de-identified ultrasound, karyotype and CMA reports will be transferred to the DCC for all enrolled patients. A study research chart should be kept, containing copies of the patient's signed consent and ultrasound reports.

4.4 Sequencing Methods

Prenatal sequencing will be performed for patients in one of the three laboratories, i.e. the Institute of Genomic Medicine (IGM) at Columbia University Medical Center, in the Human Genome Sequencing Center CLIA certified Clinical Laboratory (HGSC-CL) at Baylor College of Medicine, and at the High Throughput Sequencing Facility at the University of North Carolina Chapel Hill.

Sequencing will be performed on an Illumina NovaSeq 6000 system. All positive sequencing results will be confirmed using Sanger sequencing and reported by a CLIA certified laboratory. It is possible that non-parentage (maternity or paternity) may be suspected through initial genomic analysis. If there is suspicion that the parent of the fetus is not the biological parent, further analysis of the genomic data will be suspended and the family will be informed they are not eligible for study participation. This approach will be addressed during the informed consent process.

Data quality control, including alignment, variant calling, filtering and prioritization will be performed using best practices refined at the local institutions.

4.5 Variant Evaluation and Return of Results

All candidate variants will first be evaluated locally at each site by a team including the site PI who is a board certified clinical geneticist, a study genetic counselor, a variant curation scientist, and a board-certified molecular geneticist with clinical genome sequencing experience to determine candidate variants relevant to the clinical phenotype. Factors used in this evaluation include the ACMG classification of the variant, whether its inheritance mode is consistent with the clinical condition, and the extent of phenotypic overlap with previously reported cases. Initially, candidate variants will be reported in the coding regions only; however it is anticipated that over the course of the study this may change to also incorporate reporting in non-coding regions.

Results that are considered pathogenic or likely pathogenic by the local team will be confirmed by Sanger sequencing and reported to genetic counselor and principal investigator who will relay the results to the provider and the family. In cases in which time is critical (e.g. preterm labor, impending fetal procedure, fetal compromise) and a causative variant is suspected, the genetic counselor/principal investigator may report results either verbally to the provider as a “research finding” or as a written preliminary result report prior to Sanger confirmation.

Compelling variants in novel genes that are not yet disease associated will be classified as variants of uncertain significance (VUS) with the qualification that they are in “genes of uncertain clinical significance (GUS)”. For compelling variants in known disease genes that have not yet been associated with this disease phenotype (also classified as VUS), in-house databases will be checked for similar variants, automated PubMed alerts will be set-up to be notified of new publications, and tools such as GeneMatcher will be used to help find other individuals with variants in the same gene. Uncertain variants will be Sanger confirmed and reported to the patient based on the judgment of the local team after consultation with the Oversight Committee. To build consensus in interpretation, the local Variant Interpretation teams will have video conferences on which uncertain results will be discussed.

For all reportable results, a copy of the Sanger confirmation report and additional relevant information as applicable will be prepared by the local team and returned to the clinical study genetic counselor who will share the result with the ordering MFM/OB physician and place it in the electronic medical record (EMR). The ordering MFM/OB physicians and the study genetic counselor will report the results to the family and discuss the implications for clinical care. Study clinical geneticists will be available for consultation. Results will also be available to the neonatal care team for use after delivery. A copy of the de-identified report will be transferred to the DCC for all patients.

Local site requirements will dictate the reporting (if at all) of ACMG secondary or other incidental

findings of the parents and fetus. Because of the stressful nature of receiving the fetal diagnosis, discussions of medically actionable secondary findings that will be reported will be deferred until a formal postpartum genetic counseling consultation, anticipated to occur within 6 weeks of infant discharge or death with the exception of findings in the fetus that may impact treatment decisions on the newborn which will be reported immediately.

Carrier status for Mendelian disorders in either parent will be reported according to the local site requirements.

Parents will be informed of negative fetal sequencing results by way of a "research" report since negative results will not be Sanger confirmed.

4.6 Study Procedures

Enrolled women will be followed from screening through 12 months postpartum unless a positive and casual CMA is reported prenatally or postnatally. Data will be collected at the following time points:

Table 2. Summary of Data Collection

One month after discussion of study results or 8 weeks after enrollment for the unsequenced prenatal group	• Maternal and paternal psychosocial survey
Delivery and neonatal care	• Pregnancy complications & delivery outcomes • Neonatal phenotype and outcomes • Coordinator review of management changes done by chart review • Physician surveys assessing change in management (sequencing group only) • Results of clinical care ordered genetic testing for unsequenced group including karyotype/CMA and any subsequent sequencing Reports of healthcare utilization and charges (prenatal) from diagnosis of fetal anomaly to delivery • Healthcare utilization and charges from delivery until discharge or death (neonatal)
One month post discharge	• Maternal and paternal psychosocial survey
12 months postpartum	• Maternal and paternal psychosocial survey, including maternal brief survey of healthcare utilization • Participant phone interview to document infant weight and length by maternal self-report and child development measures using the Ages and Stages questionnaire (ASQ-3)

Neonatal phenotypes will be re-evaluated within one week of birth to identify additional clinical features. If significant new clinical findings are discovered, the sequencing variants will be re-evaluated immediately by the local team and any new diagnoses confirmed and reported by the study staff to the neonatologist or other appropriate care provider who will report this to the family in concert with the study coordinator. A copy of de-identified genetic reports (CMA and/or sequencing) will be transferred to the DCC for all enrolled patients in the unsequenced group.

4.7 Psycho-Social Surveys

Each participant (both members of the couple) will complete an online 20-minute survey at 3 time points and will receive a gift card after the last survey is completed. The three time points are:

- One month after disclosure of study results or 8 weeks after enrollment for the unsequenced prenatal group
- One month after discharge from the hospital or end of the pregnancy if there is a fetal demise or pregnancy termination
- Twelve months postpartum

The measures collected at each time period are detailed in Table 3.

Table 3. Pyschosocial Survey Measures

Variable	**Measure**	**Time**		
		Post results/ enroll	**1mth pp**	**12mth pp**
Anxiety	Personal Health Questionnaire-8	X	X	X
Depression	General Anxiety Disorder	X	X	X
Genetic Knowledge	Adapt from measures used in CSER/eMERGE	X		
Numeracy	Adapt from measures used in CSER/eMERGE	X		
General Optimism	Life Orientation Test	X		
Tolerance with Ambiguity	Tolerance with Ambiguity	X		
Perceived control over health	Internal Health Locus of Control	X		
Genetic Essentialism	Parrott Genetic Essentialism	X		
Consenting and Education Experience	Modified Genetic Counseling Satisfaction Scale and Doctor-Patient perceptions of communication	X		
Satisfaction w/ decision to Participate	Decision Regret Scale	X	X	X
Satisfaction w/ decision to continue	Decision Regret Scale	X	X	X
Parent-Infant relationship	Parent-Infant Attachment Questionnaire (Reck 16 question)		X	X
Parenting stress & anxiety for child	Child Vulnerability scale [54]		X	X
Parents' experience of parenting	Parent Sense of Competence Scale			X
Therapeutic Optimism/Prognosis	Brief Illness Perception Questionnaire	X	X	X
Marital Relationship	Kansas Marital Satisfaction Scale	X	X	X
Quality of life	ITQOL: https://www.healthactchq.com/surveys.php			X
Prenatal sequencing **only**				
Results Disclosure	Disclosure and non-disclosure of results to	X	X	X

and Secrecy	others			
Understanding of Results	Adapt from measures used in CSER/ eMERGE	X	X	X
Emotional Response to Results	FACToR-12 (12 items)	X	X	X
Perceptions of uncertainty of results	PUGS perceptions of uncertainties in genetic sequencing	X	X	X

The end of the survey will have a free text box for participants to add any other details they wish to share and also ask if the participant is willing to speak by phone in a semi-structured interview (Section 4.7.1).

4.7.1 In-depth Qualitative Interviews

The impact of learning about the prenatal WGS test and return of prenatal WGS results will be evaluated by interviewing 45 sets of parents (90 participants) one month after result disclosure or an equivalent time for those who decline testing, after discharge from the hospital, and 12 months after delivery. Those willing to be interviewed will indicate this at the end of their psychosocial survey. Depending on the number of couples who volunteer for an interview, research staff will purposefully sample a range of parental ages, ethnicities, educational level, site, and fetal anomaly. Participants with positive or uncertain genetic test results and those who had difficulty adjusting to the information or were dissatisfied with participation in the study and have decision regret will be oversampled. Interviews at each time point will focus on the following:

- The interview after results disclosure will focus on educational needs for consenting to the study, how results are disclosed, how much information is provided and by whom, understanding of the results, and support needed during the pregnancy after results are provided.
- The interview after discharge will focus on how the results of the genomic test influenced decisions about medical management including delivery, neonatal care, planning, connecting with other families with the same condition, disclosure of results to others, and impact on interpersonal relationships.
- The interview 12 months after delivery will focus on impact of fetal genomic results on longer-term management, perceptions of the child and potential vulnerability and impact on family relationships.

Both parents will be interviewed since men are often not included in these studies and are an integral part of the decision-making process and long-term outcome of the family. The interview data will be used to develop recommendations and identify factors associated with decision satisfaction, and to develop recommendations for pre-and post-test genetic counseling that reflect the needs of women and their partners as they make decisions about undergoing prenatal sequencing, or continuing a pregnancy after learning about a known or uncertain fetal sequencing abnormality.

4.8 Healthcare Utilization and Change in Management

Information on healthcare utilization and charges will be collected starting at the time of the diagnosis of the fetal anomaly through discharge from the hospital postpartum. Healthcare utilization will be abstracted from the prenatal and neonatal records and include both outpatient and inpatient visits, laboratory tests, imaging tests, invasive testing, prenatal and postnatal surgery, primary care visits, specialty care visits, diagnostic procedures, and medications. In addition, site coordinators will work with hospital billing to obtain detailed billing records from delivery admittance through discharge for each study participant, including the hospital charge ratio. In the 12-month participant psychosocial survey,

information will also be recorded on healthcare utilization post neonatal discharge, including number of infant hospitalizations, surgeries, procedures and specialist visits.

To identify how sequencing results changed the original care plan, the site coordinators will review all prenatal and postnatal medical records from diagnosis until discharge or death. Specifically, the clinical diagnosis, anticipated prognosis, procedures, medications, diet, surgery, non-routine testing and imaging will be documented. Physician surveys evaluating change in clinical management (i.e., the reasons and timing for management decisions and whether they were influenced by a genetic test result) will be completed by both the MFM/OB for prenatal assessment and a designated neonatal fellow in consultation with the clinical care team at each institution. The survey will include questions focused on clinical utility, such as: 1) have there been any changes to the patient's treatment plan based on the sequencing results, 2) have there been any changes to the way that you counsel the patient/family regarding the immediate medical management as a result of the sequencing results, and 3) have there been any changes to the way that you counsel the patient/family regarding the long term medical management as a result of the sequencing results. The care and the results of the surveys will be presented and reviewed by the Clinical Adjudication Committee which will determine whether an alteration in health care management occurred based on sequencing results.

4.9 Adverse Event Reporting

Detailed information concerning adverse events assessed to be definitely, probably or possibly related to study procedures will be collected and evaluated throughout the conduct of the study. Adverse events will be reported to the Data and Safety Monitoring Board.

4.10 Study Outcome Measures and Ascertainment

4.10.1 Primary Outcome

1. Pathogenic, likely pathogenic, or VUS variants identified by sequencing and deemed reportable by the Variant Adjudication Committee
2. Healthcare costs from time of diagnosis of anomaly to infant discharge between sequenced and unsequenced groups

4.10.2 Secondary Outcomes (Prenatal sequencing and unsequenced prenatal)

1. Perinatal outcomes including gestational age at delivery, major morbidities including length of ventilator support, sepsis, need for pressor support, need for ECMO, metabolic abnormalities (e.g., acidosis, elevated uric acid, hypo-/hyperglycemia), intraventricular hemorrhage/periventricular leukomalacia, encephalopathy, and seizure
2. Neonatal/infant death at time of discharge and at 12 months of age
3. Length of initial NICU stay and number of days spent in the hospital between initial discharge and 12 months of age
4. Infant weight and length at 12 months of age
5. Developmental parameters (communication, gross motor, fine motor, problem solving and personal-social) at 12 months of age using ASQ-3
6. Anxiety following result disclosure (or 8 weeks post enrollment for the unsequenced group), neonatal discharge and 12 months postpartum
7. Depression following result disclosure (or 8 weeks post enrollment for the unsequenced group),

neonatal discharge and 12 months postpartum

8. Quality of life for the patient and family at 12 months postpartum
9. Incremental cost per Quality Adjusted Life Year (QALY)

4.10.3 Secondary Outcomes (Prenatal sequencing only)

10. Apparent prenatal phenotypic expansion from currently defined pediatric phenotypes
11. Variants of uncertain significance (VUS) that have not yet been associated with this disease phenotype
12. VUS subclassified as compelling variants in novel genes that are not yet disease associated (genes of uncertain clinical significance; GUS)
13. Pathogenic, likely pathogenic and VUS variants identified by sequencing (coding and non-coding regions) compared with coding regions only (digital WES)
14. Pathogenic, likely pathogenic and VUS variants identified by analysis of a proband alone compared to a proband-parent trio
15. Change in management decisions attributable to genomic results defined as changes to the patient's treatment plan or changes to the counseling of the patient/family regarding the immediate or long-term medical management
16. Accuracy of parental understanding of genetic test results
17. Educational/counseling and social support needs of the mother and father
18. Changes in classification of sequencing variants over time
19. Turnaround time of sequencing components and how it changes over time.

5 Statistical Considerations

5.1 Power and sample size

The first primary outcome for this study is the frequency of pathogenic, likely pathogenic, or VUS genomic variants identified by sequencing among participants with a normal karyotype and CMA. The sample size is based on the incremental yield reported in a prospective study of WES performed at Columbia University in a similar population.[55] Among 234 parent-fetus trios without CMA abnormalities, a genetic diagnosis was reported in 22 (9.4%). The precision of the estimate for a range of sample sizes and estimates is shown in Table 4. An estimate of 9.4% and sample size of 750 in the prenatal sequenced group will have a precision of 2.3% (confidence interval half-width) with a 93% probability.

Table 4. Precision for range of estimates and sample sizes with at least 90% probability

Estimate	Sample size	CI ½-width
9.0%	700	2.4%
	750	2.3%
	800	2.2%
9.4%	700	2.4%
	750	**2.3%**
	800	2.3%
9.8%	700	2.5%
	750	2.4%
	800	2.3%

The second primary outcome is healthcare costs per case from the diagnosis of an anomaly until neonatal discharge. The utilization of health care and cost only will include women that deliver at one of the study centers. Given that some of the recruiting centers include referrals in which the women will not deliver at the study center, power estimates assume cost will be available on 500 of the 750 in the prenatal sequencing group. Similarly, of the 350 unsequenced prenatal controls, 15% are assumed to have a positive pre-/postnatal CMA or deliver elsewhere resulting in approximately 300 unsequenced prenatal controls available for analysis. The total charges for deliveries at Columbia University that required a NICU admission in 2016 and 2017 (partial year) were used to provide cost estimates. The charges follow a lognormal distribution approximately with a mean total charge of $277,902 and standard deviation of $389,484. Assuming 500 participants in the prenatal WGS group, and 300 unsequenced prenatal control participants, the study will have 80% power to detect a mean ratio of 0.80 (20% reduction) with an alpha=0.05 two-sided.

The secondary analyses focused on educational, counseling and psychosocial needs will compare the prenatal sequencing (those receiving a genetic result of any type) with the unsequenced prenatal group. Assuming an 80% completion rate will result in 600 prenatal sequencing and 280 unsequenced prenatal controls. Assuming a prevalence of 30% in the unsequenced prenatal control group for binary outcomes, the study will have more than 80% power to show a 30% reduction (30% to 21%) in the prenatal sequencing group with an alpha=0.05 two-sided. For a continuous outcome, the study will have 80% power to detect a small effect size given by Cohen's d=0.2 with an alpha=0.05 two-sided.[56]

5.2 Prenatal Sequencing Group Only

The primary and secondary outcomes for the sequencing variants are descriptive and will be reported as

the observed proportion (frequency) with 95% confidence intervals. Exact confidence intervals will be reported as appropriate. Sequencing findings include variants or no variants (negative). Variants are further classified as pathogenic, likely pathogenic, or VUS. VUS is further categorized by whether the variants are in genes related to the fetal phenotype or known to cause severe childhood disease, or in a gene of uncertain significance (GUS). Sequencing findings will be reported overall, by single vs. multiple anomalies, by organ system (abdominal wall, CNS, face/ear, effusion, intrauterine fetal growth, GI tract, genitalia, heart, neck, renal tract, skeletal, spine, thorax), and by key demographics including maternal age, race/ethnicity and fetal sex. Pearson's chi-square or Fisher's Exact test for categorical variables and Mann-Whitney U test for continuous outcomes will be used to assess associations by variant classification.

For each variant meeting the primary outcome definition (pathogenic, likely pathogenic, and uncertain genomic variants), the type of test that would identify the variant (WGS only, WES) will be determined centrally and used to report the incremental number of pathogenic, likely pathogenic, and VUS variants identified by WGS compared with WES.

Change in management decisions attributable to sequencing results are descriptive and will be reported as the frequency with 95% confidence intervals overall and by variant classification.

5.3 Prenatal Sequencing and Prenatal Unsequenced Groups

Clinical management including health care utilization and cost, and pregnancy and neonatal outcomes will be compared among the prenatal sequencing group and the unsequenced prenatal group. Women whose fetus or neonate is found to have positive/causal findings on CMA will be excluded from these analyses. Initially, demographic data and severity of ultrasound findings will be compared to ensure there are no significant differences between the two study groups (prenatal sequencing and unsequenced prenatal group). If differences are found, the covariates will be adjusted for in the analyses.

Categorical variables will be reported as the number and frequency and associations assessed by the Pearson's chi-square or Fisher's Exact test. The distributions for continuous variables will be assessed for normality and transformed to fit a normal distribution if possible. Normal distributions will be reported as mean and standard deviation and compared with the t-test, and non-normal data will be reported as median and interquartile range and compared with the Mann-Whitney U test.

For each outcome, if the study groups show a significant difference, interactions will be tested and subgroup analyses conducted if the interaction is significant ($p<0.05$). Pre-specified subgroup analyses include maternal age, race/ethnicity and fetal sex.

The amount of missing data, missing data patterns, and identification of variables associated with missingness will be explored to inform the primary and sensitivity statistical analyses. Analytic techniques used to address missing data bias will be used as appropriate. A two-sided nominal p-value less than 0.05 will indicate statistical significance.

5.3.1 Healthcare utilization and cost

Direct medical costs including cost of prenatal/postnatal sequencing, medical care for the women and newborn (hospital costs, surgeries, emergency room visits, physician office visits, outpatient services, home health services, and medications) will be estimated using health care charges associated with each of these services. Unit costs for outpatient services, surgical procedures, laboratory tests, medications and consultations will be adjusted by health system specific cost-to-charge ratios. If cost-to-charge ratios are not available, published ratios or in rare cases Medicaid reimbursements will be used.

The effect or outcome measure for cost effectiveness will be quality-adjusted life years (QALYs). Maternal and neonatal QALYs will be estimated by applying published utility weights (1=perfect health,

0=death) from various health states to components of the conditions found by sequencing. Cost-effectiveness of prenatal sequencing will be evaluated as the incremental cost per QALY with a threshold of less than $100,000 per QALY as cost effective. Cost benefit analysis will also be performed and be evaluated by dividing the costs of care and outcomes from the intervention by the costs of usual care. A CBA < 1 indicates that the intervention is cost saving and thereby cost beneficial.

All estimates of costs and outcomes will be reported as means with 95% confidence intervals. Sensitivity analysis will be conducted to evaluate the impact of uncertainty on the results. Varying probabilities (e.g. baseline risk of sequencing findings, change in neonatal outcomes) and cost parameters (e.g. cost of sequencing, healthcare costs) will be used to take into account potential clinical scenarios that might deviate from the baseline estimates. One-way deterministic sensitivity analysis for individual factors will be employed and Monte Carlo simulation to assess the robustness of the findings by simultaneously sampling distributions around multiple parameters within the model and report 95% confidence ellipses and acceptability curves.

5.3.2 Psychosocial outcomes

The secondary psychosocial outcomes are descriptive and will be reported as the observed proportion (frequency) with 95% confidence intervals. Exact confidence intervals will be reported as appropriate. All outcomes will be assessed at three time periods (post disclosure, post discharge, and 12 months postpartum) and each time period will be analyzed separately. The initial analyses will compare prenatal sequencing (those receiving a genetic result of any type) with the unsequenced prenatal group. Exploratory analyses will include pairwise comparisons between each variant classification (unsequenced prenatal group, negative sequencing result, VUS result, and pathogenic/likely pathogenic sequencing results).

Associations between the outcomes and key demographics also will be assessed. Further, association between outcomes and study groups will be tested in a multivariate setting adjusting for other variables hypothesized to affect outcome (e.g. demographics, genetic essentialism and genetic optimism). Continuous outcomes will be analyzed using a multivariate linear regression model and binary outcomes using a logistic regression model. Standard model selection methods and regression diagnostics will be performed to assess goodness of fit.

A thematic analysis will be performed for the qualitative interview data to identify patterns or themes in these data. Summary statistics will be reported for the themes.

6 Data Collection

6.1 Web Data Entry Systems

A web data entry system will be set up to present data screens for the entry of the data listed below. Data will be collected on standardized forms on which most responses have been pre-coded. Data collection, including summary sequencing result data, will be either directly entered from source material and entered on the web interface or entered on case report forms for later keying on site. For collection of pre- and postnatal phenotype data, use in MIDAS of the Human Phenotype Ontology (HPO) will be incorporated. Documents such as the prenatal ultrasound and sequencing results reports will be transferred to the DCC via the MIDAS system once de-identified

The forms will be set up in 2 web-based data entry systems as described below:

- MIDAS (Multimodal Integrated Data Acquisition System) is a data entry and management system designed specifically for research studies. Data will be entered using a web interface into the MySQL database located at the Data Coordinating Center. The system allows extensive data auditing and reporting to assist users with data correction/verification as well as patient management.
- REDCap is a web-based and database-backed platform. The psychosocial survey instruments will be completed directly by study participants using REDCap software hosted by the Clinical Coordinating Center. Data will be exported regularly to the DCC.

6.1.1 Data Collection Forms

The following forms will be entered into MIDAS:

PG01: Screening Log

PG01A Lab sample tracking form

PG02: Screening Results and Eligibility Form

PG03: Prenatal Imaging Form

PG04: Baseline Data Form (includes demographics, relevant maternal history including previous pregnancy data)

PG06: Sequencing Results Summary Form

PG07: Delivery/ Neonatal Clinical Outcome Form includes outcomes through discharge

PG08: Health Management Report to be completed by attending provider(s)

PG09: Clinical Cost Form includes prenatal and pre-discharge cost

PG10: One Year Outcome Form

PG11: Ages and Stages Questionnaire (ASQ-3)

PG12: Adverse Event Form

PG13: Patient Status Form includes withdrawal status

The following form will be entered directly into REDCap by participants:

PG14: Psychosocial survey

6.2 Centralized Data Management

The DCC will monitor on an ongoing basis the acquisition, completeness and quality of data. This will include review of laboratory compliance in timeliness of reporting prenatal diagnosis testing, including the evaluation and then the reporting of sequencing results to patients, where applicable. These data will be edited for missing, out of range and inconsistent values, and queries forwarded to their point of origin for review and resolution. Reports including timelines for form completion will be generated prompting submission of outstanding data forms.

Bioinformatics files generated during sequencing will be stored initially at the sequencing laboratories and finally submitted directly to NIH repository(ies). The DCC will prepare summary and clinical data for submission to the appropriate NIH data repository. (See Data archiving)

6.3 Performance Monitoring

Site visits will be conducted by DCC staff to the recruitment centers and the sequencing laboratories, accompanied by a laboratory supervisor from another participating laboratory or a member of Variant Oversight Committee. The purpose of each site visit is to review study procedures, assess compliance with the study protocol, and assess the quality of the study data and records. A written report will be reviewed by the Steering Committee.

The DCC will also present regular reports to the Steering Committee. These include:

- Monthly recruitment reports - reports of the number of patients screened and enrolled by month and by recruitment site.
- Quarterly Steering Committee reports - a report detailing recruitment, baseline patient characteristics, data quality, incidence of missing data and adherence to study protocol by recruitment site/ sequencing laboratory.

7 WGS Pipeline Development

7.1 Evaluation of the incremental value of WGS compared to WES in understanding the etiology of birth defects

This study will provide important insights into the genetic basis of birth defects. At present, the etiology of many structural anomalies can be determined by WES and as additional genes and variants in the coding regions are related to specific phenotypes, additional causes will become known. However, studies to date strongly suggest that WES, while clinically valuable, will not identify all causes. By exploring the frequency of *de novo* variation in regulatory and other non-coding regions of the genome and evaluating their distribution in functional classes (SNVs, SVs and CNVs), additional understanding of genetic causes of developmental alterations will occur. Accordingly, this exploratory aim will comprehensively interrogate WGS data to identify the role of errors in noncoding regions in the etiology of birth defects. The aim will also explore the development of pipelines and software to improve the integration of WGS into clinical care. This will require the development of multiple variant calling tools to catalog a comprehensive set of variants including SNVs/Indels, STRs, SVs, and CNVsI from WGS.

The current variant assessment framework will be extended to include the interpretation of WGS data outside of protein-coding regions through the evaluation of: 1) Regions intolerant to variation as assessed from population genetics; 2) Cis-regulatory regions known to regulate genes that are intolerant to functional variation and to affect transcript levels or splicing; and 3) enhancer elements and other eQTLs deduced to impact expression of known intolerant genes. This will result in a comprehensive set of regulatory regions that would be especially useful in evaluating SVs and CNVs where a functional effect (such as loss of important enhancer) is easier to predict. Although similar tools have previously been used for specific research ends, this study will refine and adjust their heuristic filters to achieve optimal sensitivity and appropriateness for clinical care and expand the diagnostic yield of WGS.

Furthermore, in partnership with Illumina Inc., these tools will be benchmarked to newer implementations of variant callers that have been specifically tuned for WGS data and optimized for speed and computational efficiency. To identify intolerant regions, previous experience using population allele frequencies of standing variation will form a basis on which to quantify purifying selection. The recently developed Orion, an intolerance metric based on the difference between an observed and expected site frequency spectrum under a neutral model, showed that it could accurately identify intolerant regions devoid of functional annotation. This method will be applied on a ten-fold larger WGS cohort (N=62,000, https://bravo.sph.umich.edu/freeze5) to calculate the observed variation and estimate the expected site frequency spectrum using mutation rates under hepta-nucleotide context (a tri-nucleotide context used originally) to refine a set of intolerant regions for assessment. It is anticipated that a hepta-nucleotide context will capture selection that is likely to occur at DNA-binding motifs more optimally and therefore allow a truer estimate of regional site frequency spectrum using the neutral model. Indeed, ~80% of GWAS non-coding variants map to putative cis-regulatory elements (CREs). Synonymous and non-canonical intronic variants will be assessed using a recently developed TRaP score (shown to identify putative deleterious synonymous and intronic substitutions with >98% specificity). Data from the ENCyclopedia Of DNA Elements (ENCODE) (such as ChIP-seq, Hi-C, ChIA-Pet, and 5C-seq) will be incorporated to determine a set of regulatory elements of intolerant and haplo-insufficient genes. Finally, regulatory regions linked to intolerant genes will be incorporated using correlations between enhancer activity and gene expression across human tissues.

8 Study Administration

8.1 Organization and Funding

This study represents a collaboration of clinical geneticists, maternal fetal medicine physicians, genetic counselors, pediatric geneticists, biostatisticians, social sciences researchers, bioinformaticians and laboratory-based whole genome sequencing technical specialists.

Table 5. Participating Organizations and Roles

	Organization	Role
Clinical Coordinating Center (CCC)	Columbia University	Project leadership, management, central IRB, overall coordination, central patient follow-up site
Recruitment Sites and sequencing Laboratories	Baylor, Columbia, UNC	Recruiting for the study and performing whole genome sequencing, variant calling, interpretation, confirmation, and reporting of results
Data Coordinating Center (DCC)	George Washington University Biostatistics Center	Protocol development, data collection, oversight and analysis
Cost-effectiveness expertise	Oregon Health Sciences University	Oversight of cost analysis
Sequencing technology	Illumina	Support for sequencing

8.1.1 Clinical Coordinating Center

The Clinical Coordinating Center (CCC) is responsible for leading and overseeing all aspects of this study. In this capacity the CCC will centrally coordinate study implementation, ongoing study management, patient follow-up, and publication of the study results. The CCC will serve as the liaison between the participating study locations, the funding agency and the Data Coordinating Center.

8.1.2 Data Coordinating Center

The Data Coordinating Center (DCC) is responsible for all aspects of biostatistical design, data management, statistical analyses, and preparation of publications based on the study results.

8.2 Committees

8.2.1 Steering Committee

The Steering Committee is the policy and decision-making group, and assumes overall responsibility for the management and conduct of the study including protocol development, oversight of the conduct of the protocol, analysis and interpretation of data, and reporting results in presentations and publications. The committee will ensure that there are synergies between the components of the project and will evaluate and provide overall direction. It will also ensure that the team makes measurable progress toward stated goals, operates within budget, follows federal policies, and submits required reports in a timely manner.

8.2.2 Operations Committee

The Operations Committee is responsible for monitoring study implementation, recruitment, day to day management, quality control, coordination and efficient communications. The committee also oversees protocol training, study logistics, recruitment progress, and monitors study updates.

8.2.3 Variant Adjudication Committee

The Variant Adjudication Committee will include external experts who will assist with interpretation of variants for which consensus interpretation by the local lab directors and PIs cannot be reached. The Committee's determination will be considered the official study result.

8.2.4 Variant Oversight Committee

The Variant Oversight Committee will include study lab directors/study lead and relevant site lab members and genetic counselors from each site. The Committee will identify systematic differences between sequencing laboratories, if any, and will serve as a "learning lab" to standardize fetal sequencing results. Specific case examples will be used to illustrate processes and examine any differences.

8.2.5 Clinical Adjudication Committee

The Clinical Adjudication Committee will include experts in maternal fetal medicine, genetics and pediatrics. The Committee will review the health management reports to ascertain, without bias, whether an alteration in health care management occurred based on sequencing results.

8.2.6 WGS Pipeline Development Committee

The WGS Pipeline Development Committee which includes bioinformaticians from each site will develop a WGS pipeline for fetal diagnosis and identify new pathways.

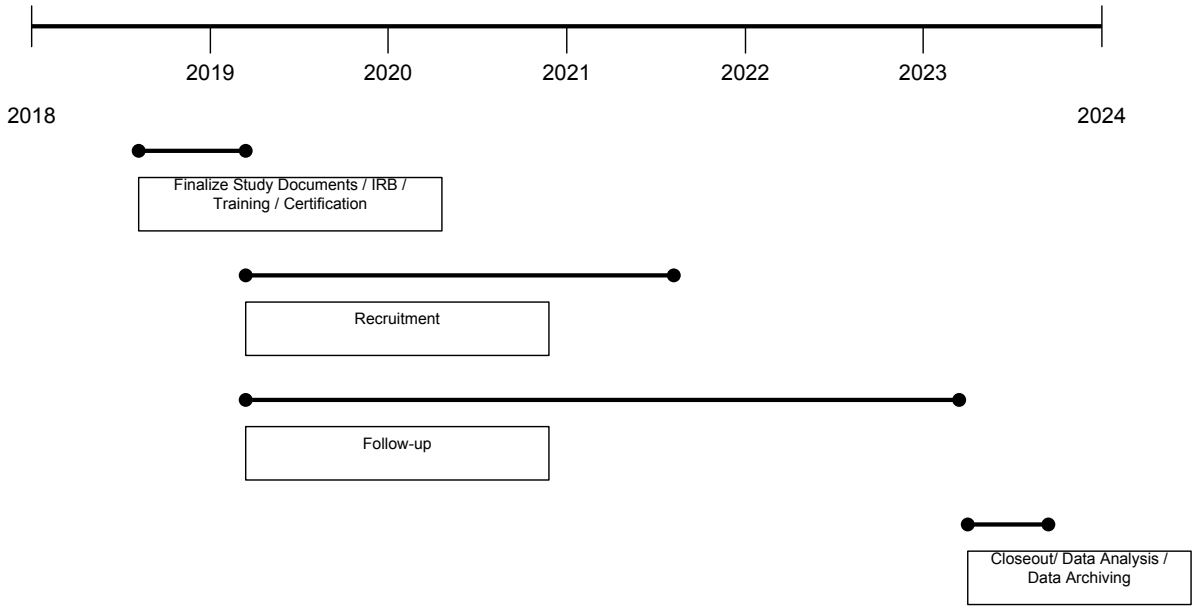
8.2.7 Data and Safety Monitoring Board (DSMB)

The DSMB will be made up of the external members of the Variant and Clinical Adjudication committees. The committee will approve the study protocol prior to initiation of the study and meet every 6 months in the course of the study. It will review interim and final reports on recruitment, and monitor logistical issues and adverse events. The committee may also recommend protocol modification or early termination of the study due to unexpected problems.

9 Study Timetable

The timetable for the study is depicted below.

Figure 2. Timeline



9.1 Training and Certification

The DCC will collaborate with the Clinical Coordinating Center to organize a workshop to train the genetic counselors at recruitment sites and the follow-up staff at Columbia in study procedures for this study, including data form completion and web entry. The manual of operations will serve as the text for the training.

Additionally, DCC personnel will work with the Clinical Coordinating Center and Lab Practices Committee to clarify and standardize laboratory practices relating to sample handling and WGS analysis. This will include review of the electronic format for the recording of results in the MIDAS database. Data handling methods will be standardized across sites, and technical staff at the DCC will be responsible for preparing systems to achieve this.

Before the study can be implemented, each recruiting site must be certified. Certification will include successful completion of the training session.

9.2 Recruitment and Data Collection Period

Educational materials will be developed targeted to participants and referring physicians.

There are 1,800 pregnancies/year with structural anomalies eligible for enrollment in the study at the three centers. Assuming that 40% of these will undergo diagnostic testing, and 25% will have an abnormal karyotype or CMA, and 50% of those eligible will consent to sequencing, it is estimated that

approximately 270 patients per year or 23 patients will be enrolled per month, with overall recruitment of 750 patients completed in ~33 months. The 350 controls would be recruited within the same time period.

9.3 Final Analysis

After a two-month period for completion of data entry and cleaning of the database for the recruitment portion of the main study, the recruitment/results sections of the data set will be locked and available for analysis. At the end of the 12 month postpartum follow-up period, approximately three months will be required to complete the final report to the Steering Committee and to submit the study's primary report for publication.

9.4 Data Archiving and Sample Storage

9.4.1 Data Archiving

At the conclusion of the study, the final dataset will be prepared by the DCC for archiving and sharing. Full de-identification of the data will be undertaken prior to submission to an NIH or public repository, including allocation of random record IDs replacing the Study ID used in the course of the study. Dates are converted to days and consideration is given to appropriate handling of unique aspects of clinical data which may serve to identify individuals. Genomic sequencing VCF files will be de-identified in a similar manner and the appropriate random ID to match the clinical record will be applied. Laboratories will be responsible for depositing any large sequencing files in the requisite repositories directly.

9.4.2 Data Sharing

In accordance with the NIH Genomic Data Sharing policy, sequencing data (including VCF files) and clinical data will be shared with other scientific investigators and through the controlled access dbGAP repository or comparable genomics commons, the Sequence Read Archive, and any NIH Birth Defects Commons that is established. A final dataset containing clinical and phenotypic data will be submitted to the NICHD data repository (DASH). In addition, new algorithms and allele frequency data will be shared with the newly developed Precision FDA platform as applicable.

9.4.3 Specimen Storage and Sharing

Remaining DNA from the trios will be stored at the local sequencing sites for secondary analyses and ancillary studies. The Steering Committee will review and approve proposals for use of the remaining DNA. The DNA will be made available to non-study investigators with appropriate IRB approval, Data Use Agreements and/or Material Transfer Agreements. Samples will be re-labeled using a random sample ID that is linked to the random record ID prior to distribution to non-study investigators.

Appendix A. Eligible Major Structural Anomalies

Abdominal wall	**Heart**
Bladder exstrophy/Epispadia	Anomalous pulmonary venous return
Body-stalk anomaly	Aortic stenosis/atresia
Cloacal exstrophy	Arrythmia
Gastroschisis	ASD
Omphalocele	AV canal defect
CNS	Coarctation
Absent or hypoplastic cerebellar vermis	Dextrocardia
Agenesis of corpus collosum	Double outlet right vertricle (DORV)
Anencephaly/Acrania	Ebsteins anomaly
Arachnoid cyst	Heart tumor
Cerebellar hypoplasia	Hypoplastic left heart
Chiari malformation	Hypoplastic right heart
Dandy-Walker malformation	Interrupted aortic arch
Encephalocele	Myxoma
Heterotopia	Pulmonary stenosis/atresia
Holoprosencephaly	Tetralogy of Fallot
Hydranencephaly	Transposition
Iniencephaly	Truncus Arteriosus
Macrocephaly (relative to fetal size)	VSD
Megalencephaly	Single ventricle
Microcephaly	Tricuspid atresia/stenosis
Lissencephaly	VSD
Parenchymal defect (gyral anomaly)	NS cardiac (abn 4-chamber view/outflow tracts, atrial/vent dilation)
Posterior fossa cyst	**Abdomen, intestine, and liver anomalies**
Spina bifida	Abnormal adrenal glands (tumor, uncertain)
Tumor	Bladder (dilated tense/floppy, ureterocele, duplex system)
Vascular anomaly	Bilateral congenital hydronephrosis
Ventriculomegaly/Hydrocephaly	Echogenic kidney
Ear	Horseshoe kidney
Anotia	Large kidney
Outer ear malformation	Multicystic kidney
Neck	Pelvic kidney
Cystic hygroma	Polycystic kidney
Teratoma	Small kidney
Eye	Multi-cystic renal dysplasia
Anophthalmia/Micropthalmos	Renal agenesis
Congenital cataract	Urethra (absent, dilated/valves)
Cyclopia	**Skeletal**
Hypertelorism	Skeletal dysplasia
Hypotelorism	Cloverleaf skull
Face	Hip dislocation/dysplasia
Facial tumor	Limb defect
Lip – Cleft	Foot (absent, oligo-/poly-/syn-dactyly, rocker bottom foot, split foot)
Genitalia	Hand (absent, brachy-/oligo-/ syn-dactyly, overlapping fingers, polydactyly (only if non-familial), split hand)
Ambiguous genitalia	
Hypospadias	Joints (fixed extended, fixed flexed)
Micropenis	Talipes
	Long bones (absent, bowed, short (<1st %ile), fracture)

Nose	**Skin**
Depressed nasal bridge	Congenital skin disorder
Palate – Cleft	Hemangioma
Abnormal profile	Tumor, unspecified
Frontal bossing	**Spine**
Micrognathia/retrognathia	Kyphosis
Gastro-intestinal tract	Sacral agenesis
Ano-rectal atresia and stenosis	Sacrococcygeal teratoma
Large bowel obstruction	Scoliosis
Small bowel obstruction	Sirenomelia
Duodenal atresia/Stenosis	**Thorax/Respiratory**
Situs abnormality	Congenital diaphragmatic hernia
Head shape	Choanal atresia
Abnormal skull shape	Congenital lung lesion/CCAM
Craniosynostosis	Hydrothorax
Abnormal calcification	Hypoplastic thorax
Effusion	Bell-shaped thorax
Hydrops	Short ribs
Ascites	**Other (reviewed centrally prior to enrollment)**
Lymphangioma	
Pleural effusion	
Skin edema	

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Prenatal Genetic Diagnosis by Genomic Sequencing (PrenatalSEQ)

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27 March 2023

Protocol Amendment History (Cumulative):

Date	Affected Section(s)	Summary of Revisions Made	Rationale
27 Mar 2023	1.1, 3.1, 3.2.3, 4.1, 4.1.1, 4.2.3, 4.6	Added enrollment of retrospective unsequenced prenatal group	Prospective enrollment of unsequenced prenatal group was not feasible. To meet sample size target, added enrollment of retrospective unsequenced prenatal group participants.
	4.7	Added additional questions to the psychosocial survey measures on COVID-19, reasons for joining the sequenced or unsequenced group, and the impact of sequencing results	To gain better understanding of the impact of these events.
	4.10	Clarified neonatal hospital charges are between delivery and infant discharge or death	Fetal deaths will not have neonatal charges
	4.10.2	Clarified secondary outcomes that will be available only on those enrolled prospectively	Retrospective unsequenced prenatal group will not have outcomes assessed at 12 months postpartum
	5.1	Removed power calculations for the secondary analysis focused on educational, counseling and psychosocial needs.	Study will not have sufficient power to evaluate since the retrospective unsequenced prenatal group will not collect these outcomes
	5.3	Clarified twelve month postpartum outcomes will be compared only among those individuals enrolled prospectively	Retrospective unsequenced participants will not collect these outcomes

22 Dec 2021	3.1, 4.4, 8.1	Clarified that there are now four labs performing sequencing for the study	Columbia has switched to using the New York Genome Center (NYGC) as their sequencing lab
	3.2.1, 3.2.2	Added the inclusion of dichorionic diamniotic twin gestations in which only one fetus has an eligible anomaly. Monochorionic diamniotic (MCDA) and monochorionic monoamniotic (MCMA) twins and a gestation in which both fetuses have an anomaly are excluded.	Improve generalizability of the results by including twin gestation. MCDA and MCMA are excluded due to the increased risk of adverse pregnancy outcomes.
	3.2.1.2, 3.2.2.2	Updated exclusion criteria to eliminate the need for delivery at a study center or associated hospital	This will allow enrollment of individuals who deliver at outside hospitals
	4.2.1	Clarified that either the twin with the anomaly or both twins will be sampled according to local clinical practice	The study only requires the twin with the anomaly to be sampled, but some sites sample both as part of clinical practice
	4.3	Clarified that data collection will include twin gestation	This will be collected in order to account for twin gestations
	4.5	Clarified that the reporting of secondary fetal findings will be per local site guidelines	This will allow sites to report secondary fetal findings during pregnancy
	4,6, 4.8	Clarified that for twin gestations, neonatal and cost data will only be collected for the twin with the anomaly	Outcomes will only be analyzed for the fetus with the anomaly

Date	Affected Section(s)	Summary of Revisions Made	Rationale
18 May 2021	3.2.1.2	Clarified exclusion criteria for the sequenced group so that only those with positive gene panels are excluded	This will allow those patients with negative gene panels to be included which could benefit from whole genome sequencing
	3.2.2.2	Clarified that CMA findings that are pathogenic or likely pathogenic as well as causal are excluded	Pathogenic or likely pathogenic variants that are related to the prenatal ultrasound finding will be excluded
	4.1	Updated eligibility flow chart to reflect changes to sample collection	Maternal buccal swab/saliva or blood may be collected
	4.2.1	Added that maternal buccal swab/saliva may be collected instead of blood	There are times when collecting blood is not feasible, and DNA can be extracted from either blood or saliva
	4.7.1	Clarified that there will be one interview between 2 weeks after results disclosure or 4 weeks after enrollment for the unsequenced group and 15 months after delivery	Each parent will be interviewed once, and the interview will be scheduled at some point during study follow-up
13 Nov 2020	3.1, 3.2.1.1, 3.2.2.1	Clarified definition of isolated nuchal translucency to be between 3.5 mm and 4.5 mm without evidence of cystic hygroma	This will distinguish between elevated nuchal translucency and cystic hygroma
	3.2.2.2, 4.2.2, 4.6, 5.1, 5.3	Clarified that those with a CMA with pathogenic variants that are related to the prenatal ultrasound finding will only be excluded if the CMA was done prior to neonatal discharge	Results obtained prior to discharge could impact cost and outcomes that are collected through discharge
	4.1	Updated eligibility flow chart to reflect changes to exclusion criteria in the unsequenced group	Only those that receive a CMA with pathogenic variants that are related to the prenatal ultrasound finding prior to neonatal discharge will be excluded
	4.2.1, 4.2.2, 4.6, 5.1, 5.3	Clarified that CMA findings must be pathogenic or likely pathogenic as well as causal	Only pathogenic or likely pathogenic variants that are related to the prenatal ultrasound finding will be excluded
	4.3	Deleted carrier screening and results, blood type, and screen from baseline data collection	No longer collected
	4.7	Clarified that the couple will receive one gift card	A gift card will be given to the couple if at least one parent completes the surveys
	6.1.1	Added the PG07B Infant Dysmorphology Form to the list of forms	Document any changes in infant phenotype assessed at birth and 12 months

Date	Affected Section(s)	Summary of Revisions Made	Rationale
13 Nov 2020	8.1	Deleted recruitment site information and clarified that Baylor, Columbia, and UNC are the sequencing labs	Baylor, Columbia, and UNC are the only sequencing labs, but additional sites may be added to improve recruitment
	9.2	Deleted three when specifying the number of centers	Additional sites may be added to improve recruitment
15 Jun 2020	3.2.1, 3.2.2	Clarified inclusion criteria for both groups to include suspected major anomalies	Some anomalies are only suspected in utero and not confirmed until after birth
	3.2.1	Added imminent delivery planned to the sequencing group exclusion criteria	Sequencing results would not be available prior to delivery if an imminent delivery is planned
	3.2.1, 3.2.2	Added intrauterine fetal demise to the exclusion criteria for both groups	Fetal death prior to enrollment was always an exclusion but not explicitly stated.
	3.2.2	Deleted negative prenatal or postnatal CMA from the unsequenced group inclusion criteria and reworded as an exclusion to exclude prenatal or postnatal CMA that are related to the prenatal ultrasound finding.	Not all infants will have postnatal CMA. Therefore, only those infants that are tested and found to have a positive will be excluded
	3.2.2	Added an inclusion to the unsequenced group to only include participants with a gestational age less than 36 weeks	Align with similar criteria in the sequenced group and to enable the psychosocial survey to be completed prior to delivery
	3.2.2	Clarified the exclusion criteria in the unsequenced group to exclude other genetic causes of the anomaly	Align with similar criteria in the sequenced group
	3.2.1, 3.2.2, 4.1.1, 4.2.1, 4.4	Changed biologic to genetic	Corrected terminology
	4.1.1	Clarified informed consent will be signed rather than written	Allows the use of electronic consenting
	4.2.2	Clarified that postpartum genetic testing is at the discretion of the clinical care team.	Not all infants in the unsequenced group will have a prenatal or postnatal CMA.
	4.3	Deleted that a research chart will be kept with copies of the consent and ultrasound reports.	All documentation is stored electronically.
	4.5	Clarified that only sequencing findings reported to the patient will be confirmed with Sanger sequencing	Some findings, if they are not causal, will not be reported to the patient. Any findings that are reported will be Sanger confirmed.

Date	Affected Section(s)	Summary of Revisions Made	Rationale
15 Jun 2020	4.6, 4.7, 4.10	Changed the timing of the first psychosocial survey to two weeks after discussion of study results or 4 weeks after enrollment for the unsequenced group	To improve the rate of survey completion and to enable the survey to be completed prior to delivery
	4.6, 4.8, 4.10, 5.1, 5.3.1	Changed the time period for maternal healthcare utilization and cost to presentation to hospital for delivery to discharge	Charge data will be collected directly from the billing data which is only available for the hospital admission.
	4.7	Added that if the patient delivers before completion of the first psychosocial survey, a survey that combines elements of the first two surveys will be administered.	Allows for completion of questions that would otherwise be missed.
	4.8	Clarified that the healthcare charge data that will be collected includes current procedural terminology medical code, description, total units and total hospital charge.	Charge data will be collected directly from the billing data and not from quantifying tests, procedures and medications.
	5.3	Added type of anomaly (lethal vs non-lethal) to the list of planned subgroup analyses	To evaluate if there is an interaction between group and type of anomaly
	6.1.1	Deleted PG09A Medication Utilization Log	Medication use will be collected directly from the billing records
	8.2.7	Changed the DSMB review to at least annually	The risk for this study is low and does not require more frequent review
	9.2	Added if recruitment falls below targets, additional sites will be added	To improve recruitment if targets are not met
	9.4.3	Clarified that remaining DNA will be stored at Columbia at the completion of the study	Samples will all be sent once follow-up is complete.
	Appendix A	Clarified typos in the list of anomalies and that only moderate to severe bilateral hydronephrosis meets eligibility	Only moderate to severe bilateral hydronephrosis would qualify as a major structural anomaly
18 Nov 2019	3.2.1	Updated inclusion criteria for prenatal sequencing group to include twin gestation reduced to singleton, either spontaneously or therapeutically, if the reduction occurred by 13 weeks, 6 days.	Early reductions are treated as a singleton pregnancy.
	3.2.1	Updated exclusion criteria for prenatal sequencing group to include other genetic causes of the fetal anomaly.	Sequencing should only be performed on participants that do not have a known cause of the anomaly.

Date	Affected Section(s)	Summary of Revisions Made	Rationale
18 Nov 2019	3.2.2	Updated inclusion criteria for prenatal unsequenced group to include twin gestation reduced to singleton, either spontaneously or therapeutically, if the reduction occurred by 13 weeks, 6 days.	Early reductions are treated as a singleton pregnancy.
	4.1	Screening flowchart was clarified to start with eligible anomalies. The flowchart for women that do not have invasive testing was clarified to include consent to the unsequenced group.	Only eligible anomalies enter the screening process. Women must consent to the unsequenced group before they enter the study flow.
	4.2.1	Added that amniotic supernatant will be stored for future research.	These samples allow for exploration of other prenatal diagnosis applications.
	4.2.1	Clarified that either the short consent for sample collection or full study consent may be signed at the screening visit.	Participants may sign either consent when they are initially approached.
	4.5	Added that any VUS that is reported to the participant will be Sanger confirmed.	All results reported to a participant must be Sanger confirmed.
	4.8	Clarified that healthcare utilization in the prenatal period will be collected from billing records when possible.	Abstraction from billing records is more efficient if they are available.
	6.1.1	Updated the name of the Adverse Event Form to include Unanticipated Problems.	Unanticipated problems must be reported to the IRB.
19 Apr 2019	3.2.1	Added prenatal sequencing or planned prenatal sequencing, including gene panels as an exclusion to the prenatal sequencing group	Women included in this assessment of prenatal sequencing should not be getting sequencing as part of clinical care as interpretation of results and outcomes may vary
	3.2.2	Added unable to consent both biologic parents as an exclusion to the prenatal unsequenced group	To be consistent with the sequenced group, as it is important to keep cohorts as comparable as possible.
	4.5	Clarified that formal notification will occur for negative fetal sequencing results only if testing is performed in a CLIA approved laboratory.	Formal reports may be given if a CLIA approved laboratory is used in testing.
	4.6	Clarified wording in Table 2 and added assessment of post-discharge genetic testing	Important to capture genetic testing that happens after discharge and up through the 12 month follow-up
	4.6	Added assessment of infant phenotype at 12 months	To ensure the results are reevaluated if new phenotypic information becomes available.

Date	Affected Section(s)	Summary of Revisions Made	Rationale
19 Apr 2019	4.8	Clarified change in management with be assessed by the physician in conjunction with the site coordinator	Change in management will require coordination between the site coordinator and the physician
	4.10.2	Changed length of ventilator support to mechanical ventilation	More clinically relevant outcome
	6.1.1	Added Infant Clinical Structural Anomalies Form PG07A	Document any changes in infant phenotype assessed at 12 months
4 Feb 2019	4.2.1	Added investigation of non-invasive prenatal testing (NIPT) to future research of maternal samples	There is an existing plan to evaluate NIPT as part of future research in the maternal samples that is detailed in the consent.
	4.5	Deleted reporting of any findings to participants prior to Sanger confirmation.	Findings must be confirmed prior to reporting unless exemption requested by IRB.
	4.5	Deleted reporting of negative findings to participants	Findings must be confirmed prior to reporting and negative findings will not be confirmed.
	6.1.1	Updated data collection forms list	New forms added
	9.4.3	Clarified sample storage includes samples for future research and that storage of all samples will be at Columbia University	A single repository is most efficient.
	4.2.1	Added investigation of non-invasive prenatal testing (NIPT) to future research of maternal samples	There is an existing plan to evaluate NIPT as part of future research in the maternal samples that is detailed in the consent.
	4.5	Deleted reporting of any findings to participants prior to Sanger confirmation.	Findings must be confirmed prior to reporting unless exemption requested by IRB.
	4.5	Deleted reporting of negative findings to participants	Findings must be confirmed prior to reporting and negative findings will not be confirmed.

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1 Introduction

1.1 Study Abstract

Whole exome and whole genome sequencing (WGS) have expanded the ability to determine the genetic etiology of previously undiagnosed disorders. This study is a multicenter cohort study to evaluate the emerging technology of sequencing for the management of fetuses with structural anomalies. The hypothesis is that a significant subset of fetal structural anomalies has a genetic etiology identifiable by sequencing and that prenatal knowledge of this information will improve perinatal care, reduce unnecessary diagnostic testing, reduce the cost of care, and improve quality of life for both the child and the family. The aims of this study are to investigate these multiple aspects of prenatal sequencing in a single study with an innovative integrated design, which will permit a robust evaluation of the benefits and risks of delivering diagnostic and prognostic genetic testing results in a prenatal setting.

The study will determine, in a sequential population of pregnancies with selected fetal structural anomalies and a negative or non-causal chromosomal microarray (CMA), the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing. To determine the impact of this information on clinical care, a control population of unsequenced pregnancies with similar structural anomalies will be prospectively recruited and the infants from both cohorts will be followed up to 1 year of age. Due to limited prospective enrollment of the controls, a portion of the unsequenced pregnancies will be enrolled retrospectively through medical record review. This study component will evaluate differences in healthcare management and cost through discharge from hospital post-delivery, and perinatal and infant outcomes through 1 year of life. The educational, counseling and psychosocial impact of sequencing results during the prenatal period, in the nursery and through 1 year of life also will be evaluated. The retrospective unsequenced pregnancies will not be included in 1-year follow-up, as they will be enrolled under a waiver of consent. Since the analytical and clinical tools needed for the full translation of sequencing into care are still developing, optimization of bioinformatic tools to improve identification of pathogenic and likely pathogenic mutations associated with prenatal phenotypes of established disease genes will be investigated, as well as identification of new genes associated with presently undiagnosed fetal/neonatal phenotypes. This study will provide an in-depth evaluation of the prenatal diagnostic value of sequencing prior to its responsible introduction into practice and will provide independent data to guide its translation.

1.2 Objectives

The main objective of this multi-center collaborative study is to evaluate sequencing (both whole exome sequencing [WES] and WGS) as a prenatal diagnostic tool in pregnancies with a structural anomaly and a negative or only non-causal karyotype/ chromosome microarray analysis (CMA). Specifically, the aims are as follows:

1. To determine in pregnancies with structural anomalies which are CMA negative (or have only non-causal findings), the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing.
    a. Examine the relative yield of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by WGS compared with analysis of the coding sequence alone (simulating WES).
2. To evaluate the impact of having sequencing results available prenatally on clinical management, including health care utilization and costs, comparing outcomes of pregnancies and neonates with *in utero* diagnosed structural anomalies that have undergone prenatal sequencing with those that have not.
3. To evaluate the educational/counseling and psychosocial needs of pregnant couples having a fetus with a structural anomaly and assessing the psychological impact of sequencing and return of results

on couples and determine the correlation with demographic factors, knowledge and attitudes about genetics, baseline parental attitudes and beliefs, and genetic test results.

4. To expand the diagnostic yield and clinical utility of fetal sequencing by developing and optimizing bioinformatic tools capable of efficiently and rapidly identifying pathogenic and likely pathogenic variants with prenatal presentations.

1.3 Purpose of the Study Protocol

This protocol describes the background, design and organization of the study and may be viewed as a written agreement among the study investigators. It is approved by the funding agency (NICHD), the study Steering Committee, the Data Safety Monitoring Board (DSMB), the single Institutional Review Board (IRB) of the Clinical Coordinating Center and participating laboratories before recruitment begins. Any changes to the protocol during the study period require the approval of the Steering Committee and the IRB.

A manual of operations supplements the protocol with detailed specifications of the study procedures.

2 Background

2.1 Introduction

With advances in prenatal imaging, increasingly there is the ability to detect birth defects prenatally and use this information to optimize perinatal and neonatal management. Simultaneously, molecular genetic diagnostics have facilitated more precise identification of the underlying cause of these birth defects and provided additional prognostic information to improve prenatal and postnatal management. The first major improvement in prenatal genomic diagnosis was chromosome microarray analysis (CMA) which identifies copy number variants (CNVs) of 100 kb to 10Mb that are below the resolution of most karyotypes. A multicenter study evaluating the clinical utility of prenatal CMA reported that CMA identified the same aneuploidies and unbalanced rearrangements as karyotyping but also identified additional, clinically significant cytogenetic information.[1] Among women with a normal karyotype, CMA identified clinically relevant deletions or duplications in 6.0% with a fetal structural anomaly and in 1.7% of those with indications of advanced maternal age or positive aneuploidy screening results. The frequency of CNVs varied by anomaly, with the highest frequency (16%) occurring in fetuses with cardiac defects.

Congenital anomalies affect 2-4% of all infants and are responsible for 20% of perinatal deaths.[2] Prenatal genetic evaluation of fetal anomalies is limited to karyotype and CMA performed on amniocytes or chorionic villi. The karyotype is abnormal in 20-25% and CMA is abnormal in 5-10% of fetal anomalies, leaving 60-70% without a genetic diagnosis.[3] WGS is the process of determining the complete DNA sequence of an organism's genome at a single time and includes both the coding and non-coding regions. Whole exome sequencing (WES) analyzes only the coding regions of the genome (exons; DNA code for making proteins). Until recently, researchers have elected to use WES because it costs less and because the functional consequences of positive findings occurring within coding regions of genes are much easier to interpret. Because of its high cost and incomplete knowledge about the role of non-coding variants in disease, WGS has primarily been used as a research tool. This may change as sequencing costs decrease and knowledge of the functional consequences of non-coding genetic variants increases. Numerous recent reports describe the speed and reliability of WGS in the context of rapid clinical sequencing since library preparation is not required[4-6] making WGS an advantageous technology for rapid genomic testing in the future.

Prenatal sequencing has the potential to alter the life course and reduce morbidity and mortality by guiding pregnancy management, delivery plans and/or the treatment of fetuses and newborns with some genetic abnormalities.[7] One example is *in utero* fetal intervention now performed for certain anomalies including aortic stenosis to avoid hypoplastic left heart syndrome, urinary diversion for posterior urethral valves, drainage of pleural effusions and chylothorax, surgical repair for spina bifida, tracheal occlusion for congenital diaphragmatic hernia, and amnio-infusion for renal agenesis.[8-14] For all of these, knowing their genetic etiology aids clinical management decisions by providing information about other associated medical features that affect the likelihood of success of the intervention. In addition, prenatal diagnosis of some metabolic conditions could guide early postnatal treatment and avoid metabolic crises that can lead to long term neurocognitive deficits. For example, pyruvate dehydrogenase deficiency can present prenatally with structural anomalies and is modifiable by a ketogenic diet and thiamine supplementation.[15,16] Finally, mesenchymal stem cell transplantation for osteogenesis imperfecta has been reported to increase skeletal mineralization and growth velocity without adverse outcomes.[17-20] With developments in gene and stem cell therapy, this type of treatment may increasingly become an option for other conditions.

2.2 Preliminary Studies

2.2.1 Postnatal Studies

WES, which analyzes the coding regions of the genome (exons) is a powerful diagnostic tool in adults and children with genetically heterogeneous conditions.[21,22] Compared with a 10% diagnostic yield with karyotype and CMA, postnatal WES has diagnostic yields of >30% in adults and children with phenotypes suggestive of monogenic disorders.[22] The mean and median overall diagnostic yields of a series of 16 studies was 36% and 38%, respectively, but was highly dependent on the *a priori* risk for a monogenic disorder. In addition to the diagnostic utility of WES in these populations, WES analysis has resulted in discovery of new disease genes with pleiotropic phenotypes.

WGS is now gaining traction as a diagnostic and discovery strategy for adults and children with a suspected genetic disorder that remains undiagnosed after WES analysis, or as a first-line approach in lieu of WES. WES includes an exon capture step, which adds time and reagent costs, and biases against coverage in GC-rich regions. WGS does not include this selection step, providing more uniform coverage which allows a lower mean read depth, offers ability to detect copy number variants (CNVs) with higher resolution than CMA and more complex balanced re-arrangements. The lower read depth however will also make it more difficult to detect mosaic mutations which are an important source of mutations in neurodevelopmental disorders including autism and epilepsy.[23,24] WGS was found to detect up to 3% of protein-coding variants missed by WES.[25] Recent WGS studies have found more causative variants in coding and non-coding regions in autism.[6] In addition, an increased burden of rare *de novo* variants in non-coding regions for congenital heart disease and diaphragmatic hernia has been seen (Chung, WC; unpublished data).

2.2.2 Prenatal Studies

Only a few case series of prenatal WES have been published to date with a wide range of indications, including pregnancy terminations, fetal demises with fetal anomalies, euploid fetuses with sonographically detected single or multiple fetal structural anomalies, and increased nuchal translucency (NT) > 3.5 mm have been reported[26,27] with widely variable diagnostic yields of 6.2 - 80%. The 2 largest series of trio exomes on over 200 fetal anomalies each had overall diagnostic rates of 6.2 and 7.5%, but was higher (14.3% and 16.0%) for fetuses with multiple anomalies.[27]

Table 1. This table describes the frequency of pathogenic variants by whole exome sequencing in various publications

First Author, Year	# Cases	Cohort Criteria	Method	Pathogenic Variants*
Normand, 2018[28]	146	Fetuses with ultrasound anomalies and a suspected Mendelian disorder	62 Trio	46/146 (32%)
Aarabi, 2018[29]	20	1 or more major structural congenital anomaly detected by ultrasound	Trio	4/20 (20%)
Fu, 2017[30]	196	Fetuses with structural abnormalities	147 Proband-only 49 Trio	34/147 (23.1%) 13/49 (26.5%)
Lei, 2017[31]	30	Fetuses with congenital anomalies of the kidney and urinary tract	23 Proband-only	3/23 (13%)
			7 Trio	1/7 (14%)

			Total	4/30 (13.3%)
Vora, 2017[32]	15	Fetuses with multiple congenital anomalies highly suggestive of an underlying genetic disorder	Trio	7/15 (47%)
Yates, 2017[33]	84	Fetuses with ultrasound abnormalities that resulted in fetal demise or pregnancy termination	29 Proband-only	4/29 (14%)
			45 Trios	11/45 (24%)
			6 Quads/4 Maternal Duos	2/10 (20%)
			Total:	17/84 (20%)
Pangalos, 2016[34]	14	Prenatal ultrasound abnormalities or malformations	Proband-only	6/14 (43%)
Alamillo, 2015[35]	7	Multiple congenital anomalies on prenatal ultrasound	Trio	4/7 (57%)
Drury, 2015[36]	24	Fetuses with an increased NT (>3.5 mm) or other ultrasound abnormality	14 Proband-only	2/14 (14%)
			10 Trio	3/10 (30%)
			Total	5/24 (21%)
Carss, 2014[37]	30	Structural abnormalities identified on prenatal ultrasound	Trio	3/30 (10%)
Yang, 2014[38]	11	Terminated fetuses with anomalies	Trio	6/11 (54%)

2.2.3 Healthcare Utilization and Cost

Integration of WES or WGS into clinical care requires not only demonstration of the ability to identify the underlying etiology of a disorder but also requires evidence that care is improved, providing value to the health care system. While WGS is currently a relatively expensive test (≥$15,000 per trio), the information may lead to significant alterations in care utilization. Given that healthcare costs can be $250,000 or more in the first year of life for some complex congenital anomalies, the genetic diagnostic information may be net cost saving. Even if not cost-saving, if clinical outcomes are improved significantly, the incremental cost of WGS may be justifiable based on its cost effectiveness. Recent studies in critically ill neonates and infants have suggested that establishing a diagnosis leads to more focused management and reduction in healthcare utilization and reduced cost. Meng et al evaluated diagnostic WES for 278 critically ill infants within the first 100 days of life and found a genetic diagnosis in 36.7%.[39] These new diagnoses led to care modification in 52%, including initiation of new subspecialist care, redirection of care, changes in medication/diet, or completion of major procedures (e.g., transplant). The greatest impact was in infants receiving rapid "critical trio sequencing".[39] A meta-analysis found that change in clinical management by WGS results was 27% (4 studies with 136 children) compared with 17% by WES (12 studies and 992 children) and 6% by CMA (8 studies of 4,271 children).[40] Another study evaluating the cost-effectiveness of WES in a pediatric setting reported the mean duration of the diagnostic odyssey was 6 years, and that the diagnostic trajectory for WES performed at the initial tertiary presentation resulted in an incremental saving of $6800 per additional diagnosis compared with the standard diagnostic pathway.[41] In summary, studies have suggested that sequencing early in the disease course may provide the maximal benefit by modifying clinical management, reducing the time to diagnosis and cost.

2.2.4 Educational & Psychosocial Needs

The added prognostic information about health, treatability, life expectancy and neurodevelopmental, behavioral and cognitive function is far beyond what is learned from ultrasound findings and standard testing, and can have a powerful impact on parents. Identification of fetal genomic information has significant value to the management of the pregnancy. Ultrasound offers an anatomic phenotype but is incapable of evaluating long-term neurocognitive potential, limiting accurate prognostication and counseling. Karyotyping and CMA can offer disease specific counseling in a combined ~30-40% of detectable cases to provide couples with the information necessary to make informed reproductive decisions, leaving significant ambiguity in 60-70%. Equally important, knowledge of the genotype can direct additional fetal, neonatal and pediatric management.

Efforts to understand the psychosocial and behavioral impact of integrating genomic technologies into adult and pediatric practice are ongoing[42-44], but to date, little empirical work has been done to understand the unique challenges of applying genomic sequencing to a prenatal population.[45] Attitudes towards prenatal screening and diagnosis are influenced by ethnicity, socioeconomic status, cultural and religious beliefs, and experiences with disability.[45-49]

While WGS results may illuminate the situation when a well-known genetic condition is diagnosed, there are challenges in counseling for some of the newer genetic conditions for which less data are available. In CMA, variants of uncertain clinical significance (VUS) and those associated with variable expressivity occur in a proportion of cases with structural anomalies (5.3%) and require extensive genetic counseling.[50] Other counseling issues include the identification of adult onset disorders of both the fetus and parent. To understand attitudes and unmet needs in the adoption of CMA testing semi-structured interviews with parents and counselors were performed to evaluate pre-test counseling, reporting of results, and assessing patient and provider experiences.[47] From the patient perspective, 5 key themes were identified 1) accepting CMA testing was an easy decision to make as patients would obtain additional information on their baby's health at no additional cost, 2) patients were blindsided by the results in cases where they initially received a normal karyotype result followed by an abnormal CMA, 3) abnormal results left patients shocked, anxious, confused and overwhelmed, 4) patients needed support to manage, understand and act on the microarray results, and 5) uncertain findings were felt to be toxic knowledge and patients wished they did not receive the results. It is anticipated that the needs for prenatal sequencing will be similar to those of prenatal CMA and that prior studies will inform the educational and support materials developed for prenatal sequencing.

2.3 Rationale for the Study

Sequencing itself is a transformative technology to identify comprehensively genetic variants accounting for a phenotype, but its application to prenatal diagnosis has not been investigated prospectively in a large cohort.[22] This study population of unselected consecutive cases will be unique in that the majority of published series to date have only included select phenotypes felt to have a genetic etiology. It is possible that these studies have overestimated the frequency and underestimated the phenotypic variability of *in utero* genetic disease based upon the case ascertainment. A more comprehensive, less biased evaluation of the molecular etiology of birth defects should lead to the discovery of previously unknown genes and phenotypic associations. To date, only descriptive series of the diagnostic yield of selected prenatal WES or WGS have been reported on fetuses with a structural anomaly, but none have evaluated the benefit of a specific genetic diagnosis.[36,37,51-53]

This study will be the first to perform a cost-effectiveness analysis to evaluate whether prenatal knowledge of the genetic cause of a fetal single-gene disorder will lead to altered fetal/neonatal management, costs, and outcomes. Also important are the pertinent psychosocial effects of introducing sequencing into a prenatal setting.

Before integration into standard care, the diagnostic capabilities of prenatal WGS must mature by developing new bioinformatic tools to specifically facilitate accurate and efficient prenatal interpretation and novel gene discovery. This can be accomplished initially by analyzing and reporting the variants from the coding regions of the genome and then subsequently analyzing the non-coding regions, allowing comparison of the differences in diagnostic yield. The current missing pieces are insufficient WGS data in fetuses with abnormalities, the lack of variant analysis algorithms tuned to the prenatal setting, and insufficient publicly deposited data to inform clinical care for prenatal genomic testing and rare developmental disorders. As more data become available (WGS data from adults, more WES/WGS from more diverse communities, Human Cell Atlas data, and ATAC seq data from appropriate times in development), it will increasingly be possible to interpret the coding and noncoding regions that are relevant to developmental disorders.

3 Study Design

3.1 Design Summary

This multicenter, observational cohort study will evaluate prenatal sequencing among pregnancies with fetal structural anomalies recruited at university based medical centers and evaluated at four genetic laboratories. A total of 1,100 pregnancies with fetal structural anomalies and meeting eligibility criteria will be enrolled into the study. Of these, 750 will undergo prenatal genomic sequencing (prenatal sequencing group) and the remaining 350 pregnancies will not have any prenatal genomic sequencing (unsequenced prenatal group). Enrollment of pregnancies with an isolated nuchal translucency measurements ≥ 3.5 mm and < 4.5 mm without evidence of cystic hygroma will be restricted to 5% within each group (sequenced and unsequenced) and isolated estimated fetal weight <5th %ile also will be restricted to 5% for each group (sequenced and unsequenced).

The prenatal sequencing group will be used to determine the frequency of pathogenic, likely pathogenic, and uncertain genomic variants identifiable by sequencing and the relative yield of sequencing. The prenatal sequencing group will be compared with the unsequenced prenatal group (prospective and retrospective) to evaluate health care management, health care utilization and cost, and perinatal outcomes. The prenatal sequencing group will be compared with the prospective unsequenced prenatal group to evaluate psychosocial needs of pregnant couples. Mothers, fathers and infants will be followed through 1 year postpartum for participants enrolled prospectively.

3.2 Eligibility Criteria

3.2.1 Prenatal sequencing group

3.2.1.1 Inclusion Criteria

1. Fetus identified by ultrasound and/or MRI with at least one of the following:
    a. One or more suspected major structural anomalies (Appendix A)
    b. A nuchal translucency measurement of ≥ 3.5 mm and < 4.5 mm without evidence of cystic hygroma
    c. A fetus less than 24 weeks 0 days gestation with normal anatomy and sonographically estimated fetal weight <5th %ile without maternal hypertension, type I diabetes, or other maternal disorders known to alter fetal growth.
2. Negative prenatal CMA (or those with CMA findings not related to the ultrasound finding)
3. Singleton gestation or dichorionic diamniotic (DCDA) twin gestation.
4. Gestational age less than 36 weeks, 0 days to allow for availability of sequencing results before delivery

3.2.1.2 Exclusion Criteria

1. Imminent delivery planned (within 3 weeks of enrollment) as sequencing results would not be available prior to delivery
2. Prenatal sequencing or planned prenatal sequencing performed outside of the study. Gene panels may be performed prior to enrollment but the results must be known and negative to be eligible (positive or unknown results are an exclusion).

3. Maternal or paternal age less than 18 years old
4. Proven infectious, teratogenic, or other genetic cause of fetal anomaly
5. For twin gestation, anomalies in both fetuses (NT, IUGR, or structural anomaly)
6. Monochorionic diamniotic (MCDA) and monochorionic monoamniotic (MCMA) twins as well as higher-order multifetal gestations
7. Planned termination of the fetus or intrauterine fetal demise (IUFD)
8. Unavailable blood or saliva samples from both genetic parents prior to sequencing
9. Parental unwillingness to participate in 1 year postnatal follow-up
10. Language barrier (non-English or Spanish speaking)
11. Previous consent to the unsequenced prenatal group or enrollment in a previous pregnancy

3.2.2 Prospective unsequenced prenatal group

3.2.2.1 Inclusion Criteria

1. Fetus identified by ultrasound and/or MRI with at least one of the following:
    a. One or more suspected major structural anomalies (Appendix A)
    b. A nuchal translucency measurement of ≥ 3.5 mm and < 4.5 mm without evidence of cystic hygroma
    c. A fetus less than 24 weeks 0 days gestation with normal anatomy and sonographically estimated fetal weight <5th %ile without maternal hypertension, type I diabetes, or other maternal disorders known to alter fetal growth
2. Declined prenatal sequencing
3. Singleton gestation or dichorionic diamniotic (DCDA) twin gestation.
4. Gestational age less than 36 weeks, 0 days

3.2.2.2 Exclusion Criteria

1. Prenatal or postnatal (prior to neonatal discharge) CMA with pathogenic or likely pathogenic variants that are related to the prenatal ultrasound finding
2. Maternal or paternal age less than 18 years old
3. Proven infectious, teratogenic, or other genetic cause of fetal anomaly
4. Positive prenatal NIPT screening for trisomy 21, 18 or 13. Positive 22q11.2 prenatal NIPT testing with consistent ultrasound findings is also an exclusion.
5. For twin gestation, anomalies in both fetuses (NT, IUGR, or structural anomaly)
6. Monochorionic diamniotic (MCDA) and monochorionic monoamniotic (MCMA) twins as well as higher-order multifetal gestations.
7. Planned termination of the fetus or intrauterine fetal demise (IUFD)
8. Unable to consent both genetic parents
9. Parental unwillingness to participate in 1 year postnatal follow-up

10. Language barrier (non-English or Spanish speaking)

3.2.3 Retrospective unsequenced prenatal group

Any changes to the prospective unsequenced prenatal group criteria are italicized below.

3.2.3.1 Inclusion Criteria

1. Fetus identified by ultrasound and/or MRI with at least one of the following:
    a. One or more suspected major structural anomalies (Appendix A)
    b. A nuchal translucency measurement of ≥ 3.5 mm and < 4.5 mm without evidence of cystic hygroma
    c. A fetus less than 24 weeks 0 days gestation with normal anatomy and sonographically estimated fetal weight <5th %ile without maternal hypertension, type I diabetes, or other maternal disorders known to alter fetal growth
2. *Did not pursue* prenatal sequencing
3. Singleton gestation or dichorionic diamniotic (DCDA) twin gestation.
4. Gestational age less than 36 weeks, 0 days *at date study-specified anomalies first diagnosed*

3.2.3.2 Exclusion Criteria

1. Prenatal or postnatal (prior to neonatal discharge) CMA with pathogenic or likely pathogenic variants that are related to the prenatal ultrasound finding
2. Maternal or paternal age less than 18 years old
3. Proven infectious, teratogenic, or other genetic cause of fetal anomaly
4. Positive prenatal NIPT screening for trisomy 21, 18 or 13. Positive 22q11.2 prenatal NIPT testing with consistent ultrasound findings is also an exclusion.
5. For twin gestation, anomalies in both fetuses (NT, IUGR, or structural anomaly)
6. Monochorionic diamniotic (MCDA) and monochorionic monoamniotic (MCMA) twins as well as higher-order multifetal gestations.
7. *Termination of the fetus or intrauterine fetal demise (IUFD) within 3 weeks of date of initial anomaly diagnosis*
8. Language barrier (non-English or Spanish speaking)
9. *Delivery at a site other than one of the study centers or associated hospitals*
10. *Unable to access prenatal or neonatal records*

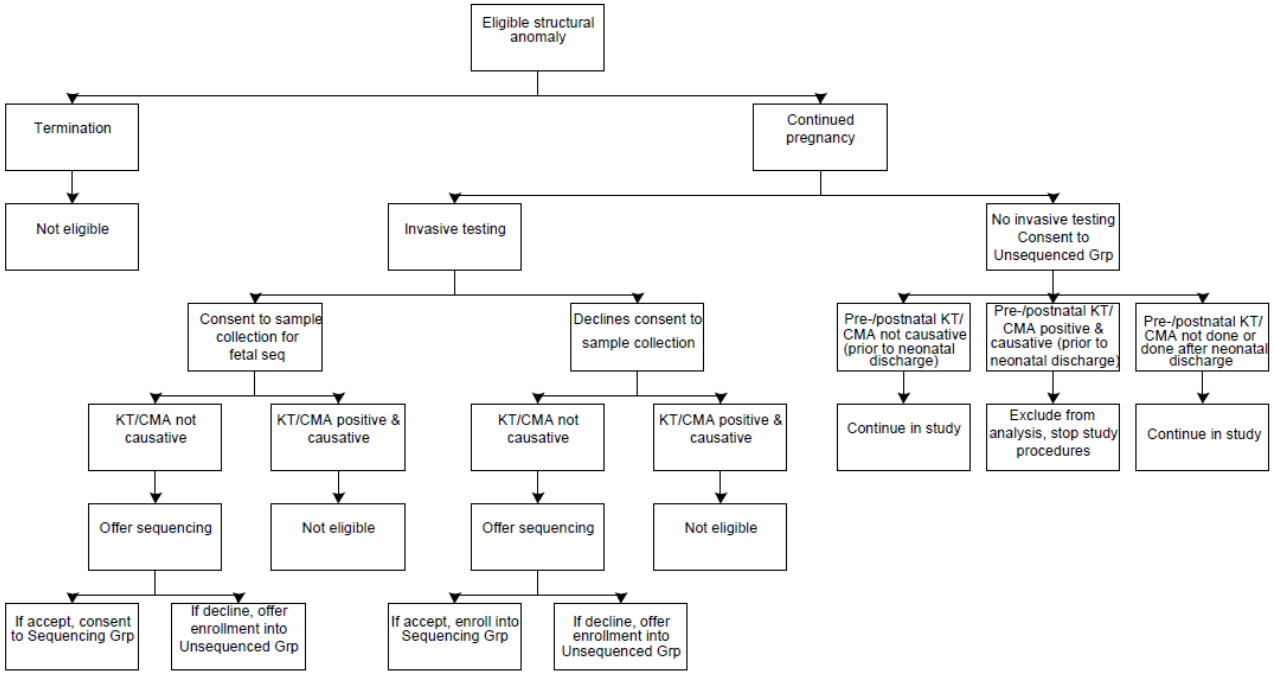
4 Study Procedures

4.1 Screening for Eligibility and Consent

Eligible patients with specified fetal structural anomalies will be identified by prenatal ultrasound and/or fetal MRI. Center investigators and their staff in the outpatient ultrasound prenatal diagnostic units will be responsible for identifying eligible patients. To maximize recruitment, coordinators at each center will screen the prenatal diagnostic logs on a daily basis to identify eligible patients.

Upon recognition of a specified fetal anomaly, the maternal fetal medicine physician (MFM)/ ultrasonographer and/or a genetic counselor will explain the ultrasound finding as per routine clinical care. At the conclusion of the initial evaluation/consult, women will be informed by the physician/counselor and/or coordinator about the study. Once the potential participant indicates willingness to hear more about the study, a study coordinator will meet with the parents, review eligibility, and explain the study. Women who choose to undergo prenatal invasive testing will be approached for the prenatal sequencing group but will not be enrolled until a negative or non-causal karyotype/CMA is confirmed (with the exception of those enrolled into expedited prenatal sequencing, section 4.2.1). Women who choose not to have any diagnostic testing or decline sequencing will be approached for participation in the unsequenced prenatal group. A portion of the unsequenced group will be enrolled through medical record review under a waiver of consent. All women with a specified fetal anomaly will be recorded on a screening log that collects screening ID, date screened, planned invasive prenatal testing, ultrasound anomaly, screening status code and reason for study ineligibility (if applicable). A participant flowchart is shown in Figure 1.

Figure 1. Prospective screening flowchart



4.1.1 Informed consent

Signed informed consent must be obtained from participants and the genetic father of the fetus prior to any study procedures for prospective enrollment. Three consents may be employed for this study:

- Short consent for potentially eligible women in the prenatal sequencing group that allows for collection of study samples prior to determination of the CMA and karyotype result. If CMA/karyotype results come back negative, eligible participants will sign the full study informed consent form to proceed with sequencing.
- Full study consent for the prenatal sequencing group that describes the specific group's participation in the study and risks
- Full study consent for the prenatal unsequenced group that describes the specific group's participation in the study and risks

Participants in both the sequenced and unsequenced groups will be assured that their participation in the study is purely voluntary and that patient care will not be affected if they decline participation in the study. Patients who are not fluent in English will be enrolled by a person fluent in Spanish. Both verbal and written informed consent and authorization will be obtained in that language; if this is not possible the patient will be excluded.

Enrollment of participants in the retrospective unsequenced prenatal group will occur under a waiver of consent.

4.2 Enrollment

4.2.1 Prenatal sequencing group

For the prenatal sequencing group, chorionic villus sampling (CVS) or amniocentesis will be performed per standard of care. For twin gestations, either the twin with the anomaly or both twins will be sampled according to local clinical practice. For CVS sampling, a portion of the villi, cultured villi or extracted DNA will be sent to the sequencing laboratory. For amniocentesis, the usual draw of 40cc of fluid will be divided by the cytogenetics lab, with a portion used for clinical testing (minimum standard amount as per diagnostic laboratory practice) and from the remainder, DNA will be extracted for sequencing in a CLIA laboratory. Additional DNA for sequencing will be extracted from the cultured cells and the sequencing lab will determine which is the optimal source for sequencing, after quality and quantity verification. Amniotic fluid supernatant will be kept for future research.

Potentially eligible participants will sign either the screening consent form or study consent form prior to collection of parental samples. Thirty ml of maternal blood or buccal swab/saliva will be collected for sequencing and future research; future research will include investigating non-invasive prenatal testing (NIPT). Paternal blood (up to 10 ml) or buccal swab/saliva will be collected for sequencing and future research. Every effort will be made to obtain samples from both parents prior to or at the time of the diagnostic procedure. If the father is absent, a sample will be obtained as soon as possible. If no paternal sample is obtained, the participant will be classified as a screen failure and will not be enrolled into the study. The parental samples will be sent for DNA extraction as soon as they become available.

If the clinical karyotype, CMA or any other non-sequencing test result is known to be positive prior to sequencing, and felt to be causative of the anomaly, the karyotype/CMA results will be recorded, the participant will be classified as a screen failure and not enrolled, and parental samples/DNA will be discarded.

If the clinical karyotype/CMA results are negative (or non-causative), eligibility criteria will be confirmed, the participant and genetic father will sign the study consent form (if not already signed),

either in person or electronically, parental samples will be collected if needed, and genome sequencing will proceed. This defines the time enrolled in the study. Parents declining participation at this time will be considered as having refused consent; these participants may be offered participation in the unsequenced prenatal group if eligibility criteria are met. Those choosing to terminate their pregnancy based on ultrasound/CMA findings are ineligible.

"Expedited" prenatal sequencing

An alternative to the prenatal diagnostic flow described above permits prenatal sequencing to be performed in parallel with the CMA, i.e. before the fetus is confirmed as CMA negative. This diagnostic workflow is at the discretion of the study research team and participants will be considered enrolled once they have signed the study consent and parental samples have been collected. If the CMA is negative or unrelated to the fetal anomaly, the case will continue to be part of the prenatal sequencing group and included in the analyses. In pregnancies in which the CMA result is found to be pathogenic or likely pathogenic (and presumed causal of the anomaly), sequencing will be discontinued or if already completed the results will be recorded and reported to the family in the appropriate manner. The karyotype/CMA results will be recorded; however, follow-up of the pregnancy through delivery will not take place, these participants will not be included in the analyses and not contribute to the sample size.

4.2.2 Unsequenced prenatal group

The prospective unsequenced prenatal group will include participants that decline invasive testing and those that plan to have invasive testing but decline sequencing (Figure 1). For participants that plan to have invasive testing but decline sequencing, enrollment into the study will occur after the clinical karyotype/CMA results are confirmed negative and the participant signs the prenatal unsequenced consent. Participants that decline invasive testing will be enrolled at the time of diagnosis of the fetal anomaly and they sign the unsequenced prenatal group consent. Women from this group who later elect invasive testing are not eligible to enroll in the prenatal sequencing group but will be included in the unsequenced prenatal analyses if the karyotype and CMA are confirmed negative or non-causal.

Postpartum genetic testing (karyotype and CMA) and genomic sequencing may be performed at the discretion of the clinical care team as part of routine care. Continued follow-up of the pregnancy and 1-year postpartum period will not take place if a pathogenic or likely pathogenic causal CMA is identified prior to neonatal discharge.

4.2.3 Retrospective unsequenced prenatal group

The retrospective unsequenced prenatal group will include participants from the screening log who did not actively decline enrollment and are otherwise eligible. Participants will be considered enrolled at the time of diagnosis of the fetal anomaly.

4.3 Baseline Data Collection

In addition to data collected for eligibility and consent, the following information will be obtained at enrollment from either medical record review or participant interview:

- Maternal and paternal: age, race, ethnicity, education, income, household composition, religion, religiosity, number of children, and family history
- Maternal medical and psychiatric history including maternal history of disorders with teratogenic risk (e.g. diabetes, teratogen exposure including medications, diseases during pregnancy (e.g. CMV, Zika)
- Obstetrical history including

- Clinical estimated date of delivery and type of conception
- Pre-procedure ultrasounds including date and detailed information on anomaly(ies)
- Number of prior pregnancies and gestational age of delivery
- Unexplained infertility
- Twin gestation

- Family history of learning disabilities, genetic disorders, congenital anomalies, stillbirths or infant deaths, significant medical problems, fetal/child structural or growth findings, consanguinity, deafness and psychiatric illnesses.
- Prenatal screening and diagnostic test results; rubella immunity status

A copy of de-identified imaging, karyotype and CMA reports will be transferred to the DCC for all enrolled patients.

4.4 Sequencing Methods

Prenatal sequencing will be performed for patients in one of the four laboratories, i.e. the Institute of Genomic Medicine (IGM) at Columbia University Medical Center, New York Genome Center (NYGC), in the Human Genome Sequencing Center CLIA certified Clinical Laboratory (HGSC-CL) at Baylor College of Medicine, and at the High Throughput Sequencing Facility at the University of North Carolina Chapel Hill.

Sequencing will be performed on an Illumina NovaSeq 6000 system. All positive sequencing results will be confirmed using Sanger sequencing and reported by a CLIA certified laboratory. It is possible that non-parentage (maternity or paternity) may be suspected through initial genomic analysis. If there is suspicion that the parent of the fetus is not the genetic parent, further analysis of the genomic data will be suspended and the family will be informed they are not eligible for study participation. This approach will be addressed during the informed consent process.

Data quality control, including alignment, variant calling, filtering and prioritization will be performed using best practices refined at the local institutions.

4.5 Variant Evaluation and Return of Results

All candidate variants will first be evaluated locally at each site by a team including the site PI who is a board certified clinical geneticist, a study genetic counselor, a variant curation scientist, and a board-certified molecular geneticist with clinical genome sequencing experience to determine candidate variants relevant to the clinical phenotype. Factors used in this evaluation include the ACMG classification of the variant, whether its inheritance mode is consistent with the clinical condition, and the extent of phenotypic overlap with previously reported cases. Initially, candidate variants will be reported in the coding regions only; however it is anticipated that over the course of the study this may change to also incorporate reporting in non-coding regions.

Compelling variants in novel genes that are not yet disease associated will be classified as variants of uncertain significance (VUS) with the qualification that they are in “genes of uncertain clinical significance (GUS)”. For compelling variants in known disease genes that have not yet been associated with this disease phenotype (also classified as VUS), in-house databases will be checked for similar variants, automated PubMed alerts will be set-up to be notified of new publications, and tools such as GeneMatcher will be used to help find other individuals with variants in the same gene. To build consensus in interpretation, the local Variant Interpretation teams will have video conferences on which uncertain results will be discussed.

Any pathogenic, likely pathogenic or VUS finding that are reported to the participant will be confirmed by Sanger sequencing. For all reportable results, a copy of the Sanger confirmation report and additional relevant information as applicable will be prepared by the local team and returned to the clinical study genetic counselor who will share the result with the ordering MFM/OB physician and place it in the electronic medical record (EMR). The ordering MFM/OB physicians and the study genetic counselor will report the results to the family and discuss the implications for clinical care. Study clinical geneticists will be available for consultation. Results will also be available to the neonatal care team for use after delivery. A copy of the de-identified report will be transferred to the DCC for all patients.

Local site requirements will dictate the reporting (if at all) of Sanger-confirmed ACMG secondary or other incidental findings of the parents and fetus. Findings in the fetus that may impact treatment decisions on the newborn will be reported immediately.

Carrier status for Mendelian disorders in either parent will be reported according to the local site requirements.

Parents will be formally informed of negative fetal sequencing results only if testing is performed in a CLIA approved laboratory.

4.6 Study Procedures

Women who are enrolled prospectively will be followed from screening through 12 months postpartum unless a pathogenic or likely pathogenic causal CMA is reported prenatally or postnatally prior to neonatal discharge. Women who are enrolled retrospectively will be followed from screening through delivery discharge. For twin gestations, neonatal data will only be collected for the fetus with the anomaly. Data will be collected at the following time points:

Table 2. Summary of Data Collection

Two weeks after discussion of study results or 4 weeks after enrollment for the unsequenced prenatal group	• Maternal and paternal psychosocial survey
Delivery and neonatal care	• Pregnancy complications & delivery outcomes • Neonatal phenotype and outcomes • Coordinator and physician review of medical records to assess change in management (sequencing group only) • Results of clinical care ordered genetic testing for unsequenced group including karyotype/CMA and any subsequent sequencing • Reports of healthcare utilization and charges from admission for delivery to discharge (maternal) • Healthcare utilization and charges from delivery until discharge or death (neonatal)
One month post discharge (prospective only)	• Maternal and paternal psychosocial survey
12 months postpartum (prospective only)	• Maternal and paternal psychosocial survey • Infant weight and length, genetic testing post discharge, and healthcare utilization by maternal self-report

	• Ages and Stages questionnaire (ASQ-3)

Neonatal phenotypes will be re-evaluated within one week of birth and infant phenotypes at 12 months to identify additional clinical features. If significant new clinical findings are discovered, the sequencing variants will be re-evaluated immediately by the local team and any new diagnoses confirmed and reported by the study staff to the neonatologist, pediatrician, or other appropriate care provider who will report this to the family in concert with the study coordinator. A copy of de-identified genetic reports (CMA and/or sequencing) will be transferred to the DCC for all enrolled patients in the unsequenced group.

4.7 Psycho-Social Surveys

Each participant enrolled prospectively (both members of the couple) will complete an online 20-minute survey at 3 time points and the couple will receive a gift card after the last survey is completed. The three time points are:

- Two weeks after disclosure of study sequencing results or 4 weeks after enrollment for the unsequenced prenatal group
- One month after discharge from the hospital or end of the pregnancy if there is a fetal demise or pregnancy termination
- Twelve months postpartum

If the patient delivers before completion of the first survey, a survey that combines elements of the first two surveys will be administered. The measures collected at each time period are detailed in Table 3.

Table 3. Psychosocial Survey Measures

Variable	**Measure**	**Time**		
		Post results/ enroll	**1mth pp**	**12mth pp**
Anxiety	Personal Health Questionnaire-8	X	X	X
Depression	General Anxiety Disorder	X	X	X
Genetic Knowledge	Adapted from measures used in CSER/eMERGE	X		
Numeracy	Adapted from measures used in CSER/eMERGE	X		
General Optimism	Life Orientation Test	X		
Tolerance with Ambiguity	Tolerance with Ambiguity	X		
Perceived control over health	Internal Health Locus of Control	X		
Genetic Essentialism	Parrott Genetic Essentialism	X		
Consenting and Education Experience	Modified Genetic Counseling Satisfaction Scale and Doctor-Patient perceptions of communication	X		
Satisfaction w/ decision to Participate	Decision Regret Scale	X	X	X
Satisfaction w/ decision to continue	Decision Regret Scale	X	X	X

Parent-Infant relationship	Parent-Infant Attachment Questionnaire (Reck 16 question)		X	X
Parenting stress & anxiety for child	Child Vulnerability scale [54]		X	X
Parents' experience of parenting	Parent Sense of Competence Scale			X
Therapeutic Optimism/Prognosis	Brief Illness Perception Questionnaire	X	X	X
Marital Relationship	Kansas Marital Satisfaction Scale	X	X	X
Quality of life	ITQOL: https://www.healthactchq.com/surveys.php			X
COVID-19	History of maternal and infant COVID-19 infection	X	X	X
Study group	Reasons for joining the sequenced or unsequenced group	X		
Prenatal sequencing **only**				
Results Disclosure and Secrecy	Disclosure and non-disclosure of results to others	X	X	X
Understanding of Results	Adapt from measures used in CSER/ eMERGE	X	X	X
Emotional Response to Results	FACToR-12 (12 items)	X	X	X
Perceptions of uncertainty of results	PUGS perceptions of uncertainties in genetic sequencing	X	X	X
Impact of sequencing results	Impact of sequencing results on pregnancy management		X	X

The end of the survey will have a free text box for participants to add any other details they wish to share and also ask if the participant is willing to speak by phone in a semi-structured interview (Section 4.7.1).

4.7.1 In-depth Qualitative Interviews

The impact of learning about the prenatal WGS test and return of prenatal WGS results will be evaluated by interviewing 45 sets of parents who were enrolled prospectively (90 participants) once between two weeks after result disclosure or four weeks after enrollment for participants in the unsequenced group and 15 months after delivery. Those willing to be interviewed will indicate this at the end of their psychosocial survey. Depending on the number of couples who volunteer for an interview, research staff will purposefully sample a range of parental ages, ethnicities, educational level, site, and fetal anomaly. Participants with positive or uncertain genetic test results and those who had difficulty adjusting to the information or were dissatisfied with participation in the study and have decision regret will be oversampled.

Both parents will be interviewed since men are often not included in these studies and are an integral part of the decision-making process and long-term outcome of the family. The interview data will be used to develop recommendations and identify factors associated with decision satisfaction, and to develop recommendations for pre- and post-test genetic counseling that reflect the needs of women and their partners as they make decisions about undergoing prenatal sequencing, or continuing a pregnancy after learning about a known or uncertain fetal sequencing abnormality.

4.8 Healthcare Utilization and Change in Management

Information on healthcare utilization and charges will be collected starting at the time of presentation to the hospital for delivery through discharge. Site coordinators will work with hospital billing to obtain detailed billing records from delivery admittance through discharge for each study participant, including the hospital charge ratio. Current Procedural Terminology (CPT) medical code, description, total units and total hospital charge will be collected. At the 12-month follow-up phone call for participants who were enrolled prospectively, information will also be recorded on healthcare utilization post neonatal discharge, including number of infant hospitalizations, surgeries, procedures and specialist visits. For twin gestations, this information will only be collected for the twin with the anomaly.

To identify if sequencing results changed the original care plan, the site coordinators will review all prenatal and postnatal medical records in conjunction with the designated physician from diagnosis until discharge or death to document change in healthcare management. Change in healthcare management will be completed by both the MFM/OB for prenatal assessment and a designated neonatal fellow in consultation with the clinical care team at each institution. The survey will include questions focused on clinical utility, such as: 1) have there been any changes to the patient's treatment plan based on the sequencing results, 2) have there been any changes to the way that you counsel the patient/family regarding the immediate medical management as a result of the sequencing results, and 3) have there been any changes to the way that you counsel the patient/family regarding the long term medical management as a result of the sequencing results. The changes in healthcare management will be presented and reviewed by the Clinical Adjudication Committee which will confirm whether an alteration in health care management occurred based on sequencing results.

4.9 Adverse Event Reporting

Detailed information concerning adverse events assessed to be definitely, probably or possibly related to study procedures will be collected and evaluated throughout the conduct of the study. Adverse events will be reported to the Data and Safety Monitoring Board.

4.10 Study Outcome Measures and Ascertainment

4.10.1 Primary Outcome

1. Pathogenic, likely pathogenic, or VUS variants identified by sequencing and deemed reportable by the Variant Adjudication Committee
2. Maternal hospital charges between time of presentation to hospital for delivery to discharge, and neonatal hospital charges between delivery and infant discharge or death

4.10.2 Secondary Outcomes (Prenatal sequencing and unsequenced prenatal)

1. Perinatal outcomes including gestational age at delivery, major morbidities including mechanical ventilation, sepsis, pressor support, ECMO, metabolic abnormalities (e.g., acidosis, elevated uric acid, hypo-/hyperglycemia), intraventricular hemorrhage, periventricular leukomalacia, encephalopathy, and seizure
2. Neonatal/infant death at time of discharge and at 12 months of age*
3. Length of initial NICU stay and number of days spent in the hospital between initial discharge and 12 months of age*
4. Infant weight and length at 12 months of age*

5. Developmental parameters (communication, gross motor, fine motor, problem solving and personal-social) at 12 months of age using ASQ-3*
6. Anxiety following result disclosure (or 4 weeks post enrollment for the unsequenced group), neonatal discharge and 12 months postpartum*
7. Depression following result disclosure (or 4 weeks post enrollment for the unsequenced group), neonatal discharge and 12 months postpartum*
8. Quality of life for the patient and family at 12 months postpartum*
9. Incremental cost per Quality Adjusted Life Year (QALY)

* Only available on participants enrolled prospectively and followed through 12 months postpartum

4.10.3 Secondary Outcomes (Prenatal sequencing only)

10. Apparent prenatal phenotypic expansion from currently defined pediatric phenotypes
11. Variants of uncertain significance (VUS) that have not yet been associated with this disease phenotype
12. VUS subclassified as compelling variants in novel genes that are not yet disease associated (genes of uncertain clinical significance; GUS)
13. Pathogenic, likely pathogenic and VUS variants identified by sequencing (coding and non-coding regions) compared with coding regions only (digital WES)
14. Pathogenic, likely pathogenic and VUS variants identified by analysis of a proband alone compared to a proband-parent trio
15. Change in management decisions attributable to genomic results defined as changes to the patient's treatment plan or changes to the counseling of the patient/family regarding the immediate or long-term medical management
16. Accuracy of parental understanding of genetic test results
17. Educational/counseling and social support needs of the mother and father
18. Changes in classification of sequencing variants over time
19. Turnaround time of sequencing components and how it changes over time.

5 Statistical Considerations

5.1 Power and sample size

The first primary outcome for this study is the frequency of pathogenic, likely pathogenic, or VUS genomic variants identified by sequencing among participants with a normal karyotype and CMA. The sample size is based on the incremental yield reported in a prospective study of WES performed at Columbia University in a similar population.[55] Among 234 parent-fetus trios without CMA abnormalities, a genetic diagnosis was reported in 22 (9.4%). The precision of the estimate for a range of sample sizes and estimates is shown in Table 4. An estimate of 9.4% and sample size of 750 in the prenatal sequenced group will have a precision of 2.3% (confidence interval half-width) with a 93% probability.

Table 4. Precision for range of estimates and sample sizes with at least 90% probability

Estimate	Sample size	CI ½-width
9.0%	700	2.4%
	750	2.3%
	800	2.2%
9.4%	700	2.4%
	750	**2.3%**
	800	2.3%
9.8%	700	2.5%
	750	2.4%
	800	2.3%

The second primary outcome is maternal and neonatal hospital charges. The utilization of health care and cost only will include women that deliver at one of the study centers. Given that some of the recruiting centers include referrals in which the women will not deliver at the study center, power estimates assume cost will be available on 500 of the 750 in the prenatal sequencing group. Similarly, of the 350 unsequenced prenatal controls, 15% are assumed to have a pathogenic or likely pathogenic causal pre-/postnatal CMA prior to neonatal discharge or deliver elsewhere resulting in approximately 300 unsequenced prenatal controls available for analysis. The total charges for deliveries at Columbia University that required a NICU admission in 2016 and 2017 (partial year) were used to provide cost estimates. The charges follow a lognormal distribution approximately with a mean total charge of $277,902 and standard deviation of $389,484. Assuming 500 participants in the prenatal WGS group, and 300 unsequenced prenatal control participants, the study will have 80% power to detect a mean ratio of 0.80 (20% reduction) with an alpha=0.05 two-sided.

5.2 Prenatal Sequencing Group Only

The primary and secondary outcomes for the sequencing variants are descriptive and will be reported as the observed proportion (frequency) with 95% confidence intervals. Exact confidence intervals will be reported as appropriate. Sequencing findings include variants or no variants (negative). Variants are further classified as pathogenic, likely pathogenic, or VUS. VUS is further categorized by whether the variants are in genes related to the fetal phenotype or known to cause severe childhood disease, or in a gene of uncertain significance (GUS). Sequencing findings will be reported overall, by single vs. multiple anomalies, by organ system (abdominal wall, CNS, face/ear, effusion, intrauterine fetal growth, GI tract, genitalia, heart, neck, renal tract, skeletal, spine, thorax), and by key demographics including maternal age, race/ethnicity and fetal sex. Pearson's chi-square or Fisher's Exact test for categorical variables and

Mann-Whitney U test for continuous outcomes will be used to assess associations by variant classification.

For each variant meeting the primary outcome definition (pathogenic, likely pathogenic, and uncertain genomic variants), the type of test that would identify the variant (WGS only, WES) will be determined centrally and used to report the incremental number of pathogenic, likely pathogenic, and VUS variants identified by WGS compared with WES.

Change in management decisions attributable to sequencing results are descriptive and will be reported as the frequency with 95% confidence intervals overall and by variant classification.

5.3 Prenatal Sequencing and Prenatal Unsequenced Groups

Clinical management including health care utilization and cost, and pregnancy and neonatal outcomes will be compared among the prenatal sequencing group and the unsequenced prenatal group (prospective and retrospective). Women whose fetus or neonate is found to have pathogenic or likely pathogenic causal findings on CMA prior to neonatal discharge will be excluded from these analyses. Twelve month postpartum outcomes will be compared only among those individuals enrolled prospectively. Initially, demographic data and severity of ultrasound findings will be compared to ensure there are no significant differences between the two study groups (prenatal sequencing and unsequenced prenatal group). If differences are found, the covariates will be adjusted for in the analyses.

Categorical variables will be reported as the number and frequency and associations assessed by the Pearson's chi-square or Fisher's Exact test. The distributions for continuous variables will be assessed for normality and transformed to fit a normal distribution if possible. Normal distributions will be reported as mean and standard deviation and compared with the t-test, and non-normal data will be reported as median and interquartile range and compared with the Mann-Whitney U test.

For each outcome, if the study groups show a significant difference, interactions will be tested and subgroup analyses conducted if the interaction is significant ($p<0.05$). Pre-specified subgroup analyses include maternal age, race/ethnicity, fetal sex, and type of anomaly (lethal vs non-lethal).

The amount of missing data, missing data patterns, and identification of variables associated with missingness will be explored to inform the primary and sensitivity statistical analyses. Analytic techniques used to address missing data bias will be used as appropriate. A two-sided nominal p-value less than 0.05 will indicate statistical significance.

5.3.1 Healthcare utilization and cost

Direct medical costs include cost of all medical care for the woman and newborn during the delivery hospitalization. These costs will be estimated using health care charges associated with each of these services. Unit costs will be adjusted by health system specific cost-to-charge ratios. If cost-to-charge ratios are not available, published ratios or in rare cases Medicaid reimbursements will be used.

The effect or outcome measure for cost effectiveness will be quality-adjusted life years (QALYs). Maternal and neonatal QALYs will be estimated by applying published utility weights (1=perfect health, 0=death) from various health states to components of the conditions found by sequencing. Cost-effectiveness of prenatal sequencing will be evaluated as the incremental cost per QALY with a threshold of less than $100,000 per QALY as cost effective. Cost benefit analysis will also be performed and be evaluated by dividing the costs of care and outcomes from the intervention by the costs of usual care. A CBA < 1 indicates that the intervention is cost saving and thereby cost beneficial.

All estimates of costs and outcomes will be reported as means with 95% confidence intervals. Sensitivity analysis will be conducted to evaluate the impact of uncertainty on the results. Varying probabilities (e.g.

baseline risk of sequencing findings, change in neonatal outcomes) and cost parameters (e.g. cost of sequencing, healthcare costs) will be used to take into account potential clinical scenarios that might deviate from the baseline estimates. One-way deterministic sensitivity analysis for individual factors will be employed and Monte Carlo simulation to assess the robustness of the findings by simultaneously sampling distributions around multiple parameters within the model and report 95% confidence ellipses and acceptability curves.

5.3.2 Psychosocial outcomes

The secondary psychosocial outcomes are descriptive and will be reported as the observed proportion (frequency) with 95% confidence intervals. Exact confidence intervals will be reported as appropriate. All outcomes will be assessed at three time periods (post disclosure/enrollment, post discharge, and 12 months postpartum) and each time period will be analyzed separately. The initial analyses will compare prenatal sequencing (those receiving a genetic result of any type) with the prospective unsequenced prenatal group. Exploratory analyses will include pairwise comparisons between each variant classification (unsequenced prenatal group, negative sequencing result, VUS result, and pathogenic/likely pathogenic sequencing results).

Associations between the outcomes and key demographics also will be assessed. Further, association between outcomes and study groups will be tested in a multivariate setting adjusting for other variables hypothesized to affect outcome (e.g. demographics, genetic essentialism and genetic optimism). Continuous outcomes will be analyzed using a multivariate linear regression model and binary outcomes using a logistic regression model. Standard model selection methods and regression diagnostics will be performed to assess goodness of fit.

A thematic analysis will be performed for the qualitative interview data to identify patterns or themes in these data. Summary statistics will be reported for the themes.

6 Data Collection

6.1 Web Data Entry Systems

A web data entry system will be set up to present data screens for the entry of the data listed below. Data will be collected on standardized forms on which most responses have been pre-coded. Data collection, including summary sequencing result data, will be either directly entered from source material and entered on the web interface or entered on case report forms for later keying on site. For collection of pre- and postnatal phenotype data, use in MIDAS of the Human Phenotype Ontology (HPO) will be incorporated. Documents such as the prenatal ultrasound and sequencing results reports will be transferred to the DCC via the MIDAS system once de-identified

The forms will be set up in 2 web-based data entry systems as described below:

- MIDAS (Multimodal Integrated Data Acquisition System) is a data entry and management system designed specifically for research studies. Data will be entered using a web interface into the MySQL database located at the Data Coordinating Center. The system allows extensive data auditing and reporting to assist users with data correction/verification as well as patient management.
- REDCap is a web-based and database-backed platform. The psychosocial survey instruments will be completed directly by study participants using REDCap software hosted by the Clinical Coordinating Center. Data will be exported regularly to the DCC.

6.1.1 Data Collection Forms

The following forms will be entered into MIDAS:

PG01: Screening Log

PG01A Lab sample tracking form

PG02: Screening Results and Eligibility Form

PG03: Prenatal Imaging Form

PG04: Baseline Data Form (includes demographics, relevant maternal history including previous pregnancy data)

PG06: Sequencing Results Summary Form

PG06A: Variant Data Log (includes mutation type, description and inheritance)

PG07: Delivery/ Neonatal Clinical Outcome Form includes outcomes through discharge

PG07A: Infant Clinical Structural Anomalies Form

PG07B: Infant Dysmorphology Form

PG08: Healthcare Management Form

PG09: Healthcare Utilization Form

PG10: One Year Outcome Form

PG11: Ages and Stages Questionnaire (ASQ-3)

PG12: Adverse Event and Unanticipated Problem Form

PG13: Patient Status Form includes withdrawal status

The following form will be entered directly into REDCap by participants:

PG14: Psychosocial survey

6.2 Centralized Data Management

The DCC will monitor on an ongoing basis the acquisition, completeness and quality of data. This will include review of laboratory compliance in timeliness of reporting prenatal diagnosis testing, including the evaluation and then the reporting of sequencing results to patients, where applicable. These data will be edited for missing, out of range and inconsistent values, and queries forwarded to their point of origin for review and resolution. Reports including timelines for form completion will be generated prompting submission of outstanding data forms.

Bioinformatics files generated during sequencing will be stored initially at the sequencing laboratories and finally submitted directly to NIH repository(ies). The DCC will prepare summary and clinical data for submission to the appropriate NIH data repository. (See Data archiving)

6.3 Performance Monitoring

Site visits will be conducted by DCC staff to the recruitment centers and the sequencing laboratories, accompanied by a laboratory supervisor from another participating laboratory or a member of the Variant Oversight Committee. The purpose of each site visit is to review study procedures, assess compliance with the study protocol, and assess the quality of the study data and records. A written report will be reviewed by the Steering Committee.

The DCC will also present regular reports to the Steering Committee. These include:

- Monthly recruitment reports - reports of the number of patients screened and enrolled by month and by recruitment site.
- Quarterly Steering Committee reports - a report detailing recruitment, baseline patient characteristics, data quality, incidence of missing data and adherence to study protocol by recruitment site/ sequencing laboratory.

7 WGS Pipeline Development

7.1 Evaluation of the incremental value of WGS compared to WES in understanding the etiology of birth defects

This study will provide important insights into the genetic basis of birth defects. At present, the etiology of many structural anomalies can be determined by WES and as additional genes and variants in the coding regions are related to specific phenotypes, additional causes will become known. However, studies to date strongly suggest that WES, while clinically valuable, will not identify all causes. By exploring the frequency of *de novo* variation in regulatory and other non-coding regions of the genome and evaluating their distribution in functional classes (SNVs, SVs and CNVs), additional understanding of genetic causes of developmental alterations will occur. Accordingly, this exploratory aim will comprehensively interrogate WGS data to identify the role of errors in noncoding regions in the etiology of birth defects. The aim will also explore the development of pipelines and software to improve the integration of WGS into clinical care. This will require the development of multiple variant calling tools to catalog a comprehensive set of variants including SNVs/Indels, STRs, SVs, and CNVs from WGS.

The current variant assessment framework will be extended to include the interpretation of WGS data outside of protein-coding regions through the evaluation of: 1) Regions intolerant to variation as assessed from population genetics; 2) Cis-regulatory regions known to regulate genes that are intolerant to functional variation and to affect transcript levels or splicing; and 3) enhancer elements and other eQTLs deduced to impact expression of known intolerant genes. This will result in a comprehensive set of regulatory regions that would be especially useful in evaluating SVs and CNVs where a functional effect (such as loss of important enhancer) is easier to predict. Although similar tools have previously been used for specific research ends, this study will refine and adjust their heuristic filters to achieve optimal sensitivity and appropriateness for clinical care and expand the diagnostic yield of WGS.

Furthermore, in partnership with Illumina Inc., these tools will be benchmarked to newer implementations of variant callers that have been specifically tuned for WGS data and optimized for speed and computational efficiency. To identify intolerant regions, previous experience using population allele frequencies of standing variation will form a basis on which to quantify purifying selection. The recently developed Orion, an intolerance metric based on the difference between an observed and expected site frequency spectrum under a neutral model, showed that it could accurately identify intolerant regions devoid of functional annotation. This method will be applied on a ten-fold larger WGS cohort (N=62,000, https://bravo.sph.umich.edu/freeze5) to calculate the observed variation and estimate the expected site frequency spectrum using mutation rates under hepta-nucleotide context (a tri-nucleotide context used originally) to refine a set of intolerant regions for assessment. It is anticipated that a hepta-nucleotide context will capture selection that is likely to occur at DNA-binding motifs more optimally and therefore allow a truer estimate of regional site frequency spectrum using the neutral model. Indeed, ~80% of GWAS non-coding variants map to putative cis-regulatory elements (CREs). Synonymous and non-canonical intronic variants will be assessed using a recently developed TRaP score (shown to identify putative deleterious synonymous and intronic substitutions with >98% specificity). Data from the ENCyclopedia Of DNA Elements (ENCODE) (such as ChIP-seq, Hi-C, ChIA-Pet, and 5C-seq) will be incorporated to determine a set of regulatory elements of intolerant and haplo-insufficient genes. Finally, regulatory regions linked to intolerant genes will be incorporated using correlations between enhancer activity and gene expression across human tissues.

8 Study Administration

8.1 Organization and Funding

This study represents a collaboration of clinical geneticists, maternal fetal medicine physicians, genetic counselors, pediatric geneticists, biostatisticians, social sciences researchers, bioinformaticians and laboratory-based whole genome sequencing technical specialists.

Table 5. Participating Organizations and Roles

	Organization	Role
Clinical Coordinating Center (CCC)	Columbia University	Project leadership, management, central IRB, overall coordination, central patient follow-up site
Sequencing Laboratories	Baylor, Columbia, UNC, NYGC	Performing whole genome sequencing, variant calling, interpretation, confirmation, and reporting of results
Data Coordinating Center (DCC)	George Washington University Biostatistics Center	Protocol development, data collection, oversight and analysis
Cost-effectiveness expertise	Oregon Health Sciences University	Oversight of cost analysis
Sequencing technology	Illumina	Support for sequencing

8.1.1 Clinical Coordinating Center

The Clinical Coordinating Center (CCC) is responsible for leading and overseeing all aspects of this study. In this capacity the CCC will centrally coordinate study implementation, ongoing study management, patient follow-up, and publication of the study results. The CCC will serve as the liaison between the participating study locations, the funding agency and the Data Coordinating Center.

8.1.2 Data Coordinating Center

The Data Coordinating Center (DCC) is responsible for all aspects of biostatistical design, data management, statistical analyses, and preparation of publications based on the study results.

8.2 Committees

8.2.1 Steering Committee

The Steering Committee is the policy and decision-making group, and assumes overall responsibility for the management and conduct of the study including protocol development, oversight of the conduct of the protocol, analysis and interpretation of data, and reporting results in presentations and publications. The committee will ensure that there are synergies between the components of the project and will evaluate and provide overall direction. It will also ensure that the team makes measurable progress toward stated goals, operates within budget, follows federal policies, and submits required reports in a timely manner.

8.2.2 Operations Committee

The Operations Committee is responsible for monitoring study implementation, recruitment, day to day

management, quality control, coordination and efficient communications. The committee also oversees protocol training, study logistics, recruitment progress, and monitors study updates.

8.2.3 Variant Adjudication Committee

The Variant Adjudication Committee will include external experts who will assist with interpretation of variants for which consensus interpretation by the local lab directors and PIs cannot be reached. The Committee's determination will be considered the official study result.

8.2.4 Variant Oversight Committee

The Variant Oversight Committee will include study lab directors/study lead and relevant site lab members and genetic counselors from each site. The Committee will identify systematic differences between sequencing laboratories, if any, and will serve as a "learning lab" to standardize fetal sequencing results. Specific case examples will be used to illustrate processes and examine any differences.

8.2.5 Clinical Adjudication Committee

The Clinical Adjudication Committee will include experts in maternal fetal medicine, genetics and pediatrics. The Committee will review the health management reports to ascertain, without bias, whether an alteration in health care management occurred based on sequencing results.

8.2.6 WGS Pipeline Development Committee

The WGS Pipeline Development Committee which includes bioinformaticians from each site will develop a WGS pipeline for fetal diagnosis and identify new pathways.

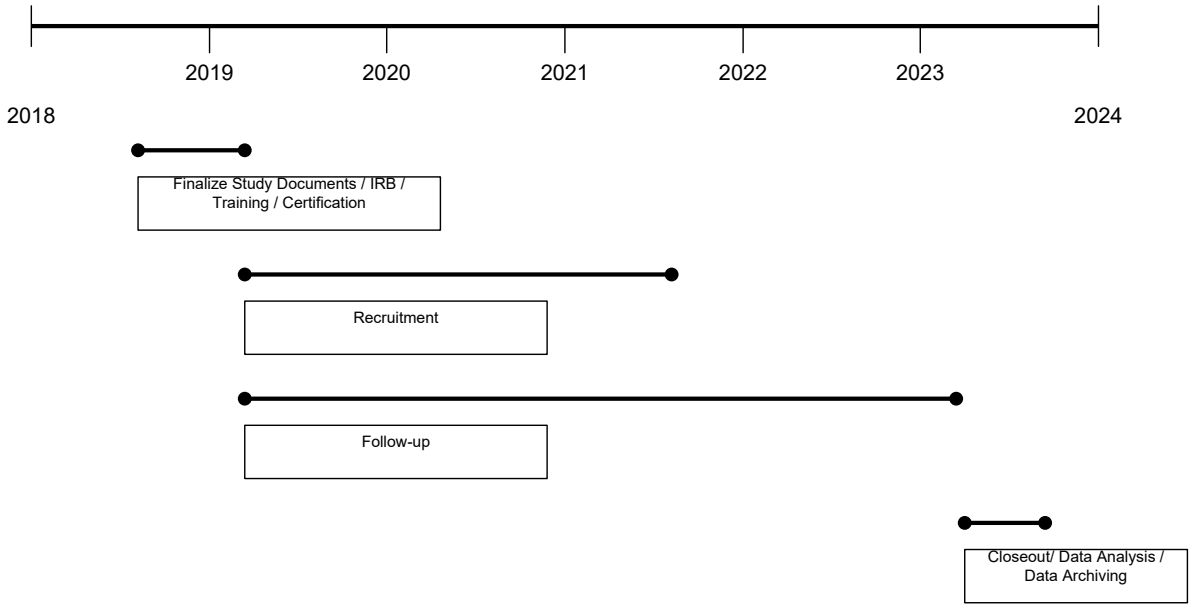
8.2.7 Data and Safety Monitoring Board (DSMB)

The DSMB will be made up of the external members of the Variant and Clinical Adjudication committees. The committee will approve the study protocol prior to initiation of the study and meet at least annually in the course of the study. It will review interim and final reports on recruitment, and monitor logistical issues and adverse events. The committee may also recommend protocol modification or early termination of the study due to unexpected problems.

9 Study Timetable

The timetable for the study is depicted below.

Figure 2. Timeline



9.1 Training and Certification

The DCC will collaborate with the Clinical Coordinating Center to organize a workshop to train the genetic counselors at recruitment sites and the follow-up staff at Columbia in study procedures for this study, including data form completion and web entry. The manual of operations will serve as the text for the training.

Additionally, DCC personnel will work with the Clinical Coordinating Center and Lab Practices Committee to clarify and standardize laboratory practices relating to sample handling and WGS analysis. This will include review of the electronic format for the recording of results in the MIDAS database. Data handling methods will be standardized across sites, and technical staff at the DCC will be responsible for preparing systems to achieve this.

Before the study can be implemented, each recruiting site must be certified. Certification will include successful completion of the training session.

9.2 Recruitment and Data Collection Period

Educational materials will be developed targeted to participants and referring physicians.

There are 1,800 pregnancies/year with structural anomalies eligible for enrollment in the study at the participating centers. Assuming that 40% of these will undergo diagnostic testing, and 25% will have an abnormal karyotype or CMA, and 50% of those eligible will consent to sequencing, it is estimated that

approximately 270 patients per year or 23 patients will be enrolled per month, with overall recruitment of 750 patients completed in ~33 months. The 350 controls would be recruited within the same time period. If recruitment falls below targets, additional sites may be added.

9.3 Final Analysis

After a two-month period for completion of data entry and cleaning of the database for the recruitment portion of the main study, the recruitment/results sections of the data set will be locked and available for analysis. At the end of the 12 month postpartum follow-up period, approximately three months will be required to complete the final report to the Steering Committee and to submit the study's primary report for publication.

9.4 Data Archiving and Sample Storage

9.4.1 Data Archiving

At the conclusion of the study, the final dataset will be prepared by the DCC for archiving and sharing. Full de-identification of the data will be undertaken prior to submission to an NIH or public repository, including allocation of random record IDs replacing the Study ID used in the course of the study. Dates are converted to days and consideration is given to appropriate handling of unique aspects of clinical data which may serve to identify individuals. Genomic sequencing VCF files will be de-identified in a similar manner and the appropriate random ID to match the clinical record will be applied. Laboratories will be responsible for depositing any large sequencing files in the requisite repositories directly.

9.4.2 Data Sharing

In accordance with the NIH Genomic Data Sharing policy, sequencing data (including VCF files) and clinical data will be shared with other scientific investigators and through the controlled access dbGAP repository or comparable genomics commons, the Sequence Read Archive, and any NIH Birth Defects Commons that is established. A final dataset containing clinical and phenotypic data will be submitted to the NICHD data repository (DASH). In addition, new algorithms and allele frequency data will be shared with the newly developed Precision FDA platform as applicable.

9.4.3 Specimen Storage and Sharing

At the completion of the study, remaining DNA from the trios and additional samples collected for future research will be stored at Columbia University for secondary analyses and ancillary studies. The Steering Committee will review and approve proposals for use of the remaining DNA and samples. The DNA and samples will be made available to non-study investigators with appropriate IRB approval, Data Use Agreements and/or Material Transfer Agreements. Samples will be re-labeled using a random sample ID that is linked to the random record ID prior to distribution to non-study investigators.

Appendix A. Eligible Major Structural Anomalies

Abdominal wall	**Heart**
Bladder exstrophy	Anomalous pulmonary venous return
Body-stalk anomaly	Aortic stenosis/atresia
Cloacal exstrophy	Arrhythmia
Gastroschisis	ASD
Omphalocele	AV canal defect
CNS	Coarctation
Absent or hypoplastic cerebellar vermis	Dextrocardia
Agenesis of corpus collosum	Double outlet right ventricle (DORV)
Anencephaly/Acrania	Ebsteins anomaly
Arachnoid cyst	Heart tumor
Cerebellar hypoplasia	Hypoplastic left heart
Chiari malformation	Hypoplastic right heart
Dandy-Walker malformation	Interrupted aortic arch
Encephalocele	Myxoma
Heterotopia	Pulmonary stenosis/atresia
Holoprosencephaly	Tetralogy of Fallot
Hydranencephaly	Transposition
Iniencephaly	Truncus Arteriosus
Macrocephaly (relative to fetal size)	VSD
Megalencephaly	Single ventricle
Microcephaly	Tricuspid atresia/stenosis
Lissencephaly	NS cardiac (abn 4-chamber view/outflow tracts, atrial/vent dilation)
Parenchymal defect (gyral anomaly)	
Posterior fossa cyst	**Abdomen, intestine, and liver anomalies**
Spina bifida	Abnormal adrenal glands (tumor, uncertain)
Tumor	Bladder (dilated tense/floppy, ureterocele, duplex system)
Vascular anomaly	Moderate/severe bilateral hydronephrosis/urinary tract dilation
Ventriculomegaly/Hydrocephaly	Echogenic kidney
Ear	Horseshoe kidney
Anotia	Large kidney
Outer ear malformation	Multicystic kidney
Neck	Pelvic kidney
Cystic hygroma	Polycystic kidney
Teratoma	Small kidney
Eye	Multi-cystic renal dysplasia
Anophthalmia/Micropthalmos	Renal agenesis
Congenital cataract	Urethra (absent, dilated/valves)
Cyclopia	**Skeletal**
Hypertelorism	Skeletal dysplasia
Hypotelorism	Cloverleaf skull
Face	Hip dislocation/dysplasia
Facial tumor	Limb defect
Lip – Cleft	Foot (absent, oligo-/poly-/syn-dactyly, rocker bottom foot, split foot)
Genitalia	Hand (absent, brachy-/oligo-/ syn-dactyly, overlapping fingers, polydactyly (only if non-familial), split hand)
Ambiguous genitalia	
Epispadias	Joints (fixed extended, fixed flexed)
Hypospadias	Talipes
Micropenis	Long bones (absent, bowed, short (<1st %ile), fracture)

Nose	**Skin**
Depressed nasal bridge	Congenital skin disorder
Palate – Cleft	Hemangioma
Abnormal profile	Tumor, unspecified
Frontal bossing	**Spine**
Micrognathia/retrognathia	Kyphosis
Gastro-intestinal tract	Sacral agenesis
Ano-rectal atresia and stenosis	Sacrococcygeal teratoma
Large bowel obstruction	Scoliosis
Small bowel obstruction	Sirenomelia
Duodenal atresia/Stenosis	**Thorax/Respiratory**
Situs abnormality	Congenital diaphragmatic hernia
Head shape	Choanal atresia
Abnormal skull shape	Congenital lung lesion/CCAM
Craniosynostosis	Hydrothorax
Abnormal calcification	Hypoplastic thorax
Effusion	Bell-shaped thorax
Hydrops	Short ribs
Ascites	**Other (reviewed centrally prior to enrollment)**
Lymphangioma	
Pleural effusion	
Skin edema	

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