

**CHROMOSOME ABNORMALITIES IN NEONATES WITH CONGENITAL HEART
DEFECTS**

by

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Congenital heart disease (CHD) contributes to the rate of birth defects in the population with an incidence of nearly 1% of all live births (1). CHD has significant Public Health importance due to its high incidence, clinical severity, and complexity of medical management. In some cases, efficiently diagnosing the underlying cause of CHD is essential to providing optimal clinical care. The etiology of CHD is hypothesized to be largely multifactorial in nature, but chromosome abnormalities account for 8-13% of all CHD (2, 3). It has recently been recognized that submicroscopic chromosome abnormalities known as copy number variants (CNVs) may also play a role in causing CHD. Array comparative genomic hybridization (array CGH) has recently been added as a first-tier test for neonates with CHD at our institution, due to its ability to detect CNVs which were previously undetectable by classical cytogenetic analysis. Our study is a prospective chart review of data from neonates with CHD admitted to the Cardiac Intensive Care Unit at Children's Hospital of Pittsburgh of UPMC. The purpose of this study was to assess the clinical efficacy of array CGH as a first-tier test in this population. Our results show that array CGH increases the detection rate of chromosome aberrations by 20% above classical cytogenetic analysis alone. However, the majority of CNVs detected in our study had unclear clinical significance. For this reason, collaboration between Cardiology and Medical Genetics is essential to interpreting the clinical relevance of array CGH results in order to provide an accurate, timely genetic diagnosis and ultimately improve the clinical care of neonates with CHD.

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PREFACE

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Nomenclature

I. Congenital heart defects

- a. AS: Aortic Stenosis
- b. ASD: Atrial Septal Defect
- c. AVSD: Atrioventricular Septal Defect
- d. BAV: Bicuspid Aortic Valve
- e. COA: Coarctation of the Aorta
- f. DORV: Double-outlet right ventricle
- g. D-TGA: D-transposition of the great arteries
- h. HLHS: Hypoplastic left heart syndrome
- i. IAA: Interrupted aortic arch
- j. LVOTO: Left Ventricular Outflow Tract Obstruction
- k. PDA: Patent Ductus Arteriosus
- l. PFO: Patent Foramen Ovale
- m. PS: Pulmonary Stenosis
- n. RVOTO: Right Ventricular Outflow Tract Obstruction
- o. TOF: Tetralogy of Fallot
- p. VSD: Ventricular Septal Defect

II. Other

- a. Array CGH: Array Comparative Genomic Hybridization
- b. CNV: Copy Number Variant
- c. DD/ID: Developmental Delay/ Intellectual Disability
- d. FISH: Fluorescence In Situ Hybridization
- e. MCA: Multiple Congenital Anomalies
- f. VUS: Variant of Uncertain Significance

1.0 INTRODUCTION

Congenital heart disease (CHD) encompasses any structural defect of the heart which is present at birth. It contributes to the rate of birth defects in the population with an incidence of nearly 1% among all live births (1). However, the incidence of moderate to severe CHD may be lower (3-6 in 1000 live births), while the true prevalence including of all types of CHD may approach 50 in 1000 live births (4-6). The incidence is also higher among premature infants (2%, excluding PDA), stillborns (3-4%), and miscarriages (10-25%) (7). There can be a wide range in the clinical severity of heart defects, but over half of all patients with CHD will receive a diagnosis within the first month of life (7). In recent years there have been remarkable advances in surgical techniques for the treatment of CHD, which have the potential to dramatically increase life expectancy and quality of life. Despite these advances, CHD is still the leading cause of mortality for children with birth defects (7), and its impact reaches into the fields of Medicine, Public Health, and Medical Genetics.

Despite many efforts to discover the etiology of CHD, the cause for the majority of cases of CHD is still unknown (8). These gaps in knowledge regarding the developmental basis of CHD have limited scientific advances in diagnosis and treatment, and can make it challenging to provide an accurate estimate of recurrence risk. Based on recurrence risks from epidemiologic studies, the majority of CHD is hypothesized to be multifactorial in origin, but several specific

causes have been identified. These include environmental factors, such as maternal diseases and teratogenic exposures, as well as genetic factors, such as chromosomal abnormalities and single gene mutations. In cases of CHD with a genetic basis, accurate and efficient diagnosis of the underlying genetic etiology is crucial to provide information about prognosis and involvement of other organ systems, as well as reproductive risks and testing options for other family members (4).

It has been estimated that between 8 and 13% of all cases of CHD are associated with chromosomal abnormalities (2, 3), but this is likely to be an underestimate of the true prevalence due to the limitations of previous studies (9). Some of these studies were large population-based studies, in which only a subset of individuals with CHD had genetic testing, often based on the presence of additional anomalies or organ system involvement suggestive of a genetic syndrome (2, 10). Therefore, many individuals with CHD were unlikely to have received chromosome analysis, as only 25 to 40% of all patients with CHD are reported to have other birth anomalies (7, 11). In addition, the detection of chromosome abnormalities was previously limited to aneuploidies or other large rearrangements detectable by classical cytogenetic analysis. For the purposes of this study, classical cytogenetic analysis refers to a G-banded karyotype. This method of chromosome analysis has been the gold standard for the detection of chromosome abnormalities, but its resolution is limited to imbalances greater than about 5 megabases (Mb) or larger (12).

In recent years, various single gene mutations and submicroscopic chromosomal rearrangements have been discovered to play an important role in causing both syndromic and isolated CHD. One well-studied example is 22q11.2 microdeletion syndrome (DiGeorge syndrome), which accounts for a significant portion (6%) of all cases of conotruncal heart

defects (13, 14). However, there is still a substantial portion of CHD, both isolated and syndromic, for which the etiology is yet to be elucidated.

With the evolution of array comparative genomic hybridization (array CGH), novel submicroscopic chromosome abnormalities have been recognized as another important cause of CHD. Array CGH can detect these submicroscopic rearrangements, also known as copy number variants (CNVs), by analyzing the genome in high-resolution in comparison with a reference genome (15). CNVs are often not detectable by classical cytogenetic analysis or targeted FISH studies. Array CGH has been recently recommended as a first-tier test, in concert with classical cytogenetic analysis, for individuals with developmental delay/ intellectual disability (DD/ID), autism spectrum disorders (ASDs), and multiple congenital anomalies (MCA) (16), but the use of array CGH for individuals with CHD has not yet been studied extensively. There have been several studies regarding the use of array CGH among individuals with CHD, but these have limited inclusion by age (fetuses, children, or adults) or by the presence or absence of other anomalies. There has been little research to date regarding the use of array CGH in a cohort of live-born neonates, including those with apparently syndromic and apparently isolated CHD. Our study has been designed to fill this gap in prior research.

At Children's Hospital of Pittsburgh of UPMC, array CGH been added as a first-tier test for infants diagnosed with CHD in the Cardiac Intensive Care Unit. However, there is still much to be learned regarding its clinical efficacy and detection rate in this population. This study was designed to answer the following questions: Does array CGH increase the detection rate of chromosome abnormalities among neonates with a congenital heart defect, in comparison with classical cytogenetic analysis alone? Are there apparent relationships between type of CHD, syndromic features, and chromosome abnormalities among our study population? Should array

CGH be used as a “first tier” test for a neonate with apparently isolated CHD or only in neonates with associated dysmorphic features or extracardiac abnormalities? Thus, the quantitative aim of this study is to compare the detection rates of classical cytogenetic analysis and array CGH. The qualitative aims of the study are to assess the clinical significance of all CNVs detected by array CGH, as well as to determine whether any relationship exists between type of CHD, extracardiac abnormalities, and chromosome abnormalities in our study population.

Our study design is a prospective chart review. Inclusion criteria for participation in the study included any neonate with a congenital heart defect and admitted to the Cardiac Intensive Care Unit (CICU) of Children’s Hospital of Pittsburgh of UPMC at less than four weeks of age. It is considered standard of care at our institution for any neonate with CHD to receive chromosome analysis and array CGH, as well as a physical exam by a geneticist to examine for dysmorphism. After obtaining consent from the parents of the patient for study participation, data collected for our study included general medical history information, physical exam results, and genetic test results. Statistical analysis of the data was performed using McNemar’s test as the primary outcome variable to analyze whether there is a significant difference between the detection rates of classical cytogenetic analysis and array CGH.

The ultimate goal of this study is to provide data which may contribute to establishing guidelines for genetic testing of neonates with a CHD. When applicable, the correct diagnosis of the genetic etiology of a congenital heart defect is critical to improving patient care by providing the family with genetic counseling information and providing the physician with guidance for medical management. The results of this study will be important to ascertain if the use of array CGH as a first-tier test for all neonates with CHD improves the efficiency and accuracy of diagnosis in this patient population.

1.1 BACKGROUND

1.1.1 Normal Cardiac Development

The process of cardiac development is a complex and dynamic process. In humans the process of cardiac development begins on days 15-16 of gestation and culminates on day 49 with the complete formation of the heart. There are five main steps of cardiac development, including the following: (8):

- 1) Days 15-16: Precardiac cells migrate from the primitive streak to assemble the cardiac crescents, also known as the cardiogenic plates.
- 2) Day 22: Formation of the primitive heart tube begins, as the cardiogenic plates fuse into one tube with two poles, the arterial pole and the venous pole. The progenitor cells of the heart tube include inner endocardial cells and outer myocardial cells.
- 3) Day 23: Cardiac looping begins as the primitive heart tube folds into a crescent shape to align future cardiac chambers. Throughout this process, cells in specific sections of the heart tube become specialized and the segments begin to differentiate into primitive chambers and transitional zones, which come together at the inner curvature of the heart tube.
- 4) Day 28: Septation and heart chamber formation begins. Formation of the septa occurs at the atrium, ventricle, and arterial pole. The transitional zones, which will give rise to the septa, conduction system, fibrous heart skeleton, and valves, are incorporated in the primitive chambers which form the atria and ventricles.

5) Until Day 49: During septation of the outflow tract, the neural crest cells enter the heart. They are incorporated into the myocardium and begin the conduction system. The two endocardial cushions of the outflow tract fuse to form the outflow tract septum and the semilunar valve. Cells from the lining of the myocardium migrate to form the coronary arterial tree. By day 49, establishment of the cardiac conduction system and coronary vasculature is complete (8).

There are four types of cardiac precursor cells which contribute to the developing heart (Figure 1). The cells of the primitive heart tube are the *first heart field*, which will eventually form the left ventricle and atria, and contribute to all other structures of the heart except for the cardiac outflow tract. The *second heart field* lies behind the heart tube; this will provide myocardial cells to contribute to the cardiac outflow tract, part of the atria, and right ventricle. The *cardiac neural crest cells* give rise to the aortopulmonary septum and great vessels. Cells of the *proepicardium* will form the epicardium and coronary vessels (17).

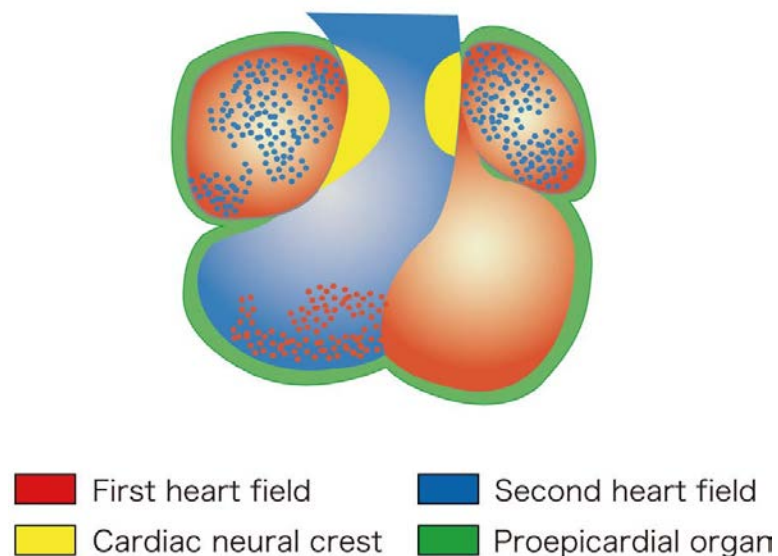


Figure 1. Precursor cells and their contribution to the four-chambered heart (17)

1.1.2 Congenital Heart Defects

Due to the complexity of the process of normal cardiac development, there are a variety of mechanisms which may contribute to defective cardiac structure or function. These include mechanical, cellular, and molecular factors. On a cellular level, the processes of growth, differentiation, and apoptosis are the basis for the entire course of cardiac development. Disruptions of these cellular processes at specific points in cardiac development have been documented to cause defects in cardiac looping, septation, chamber formation, and arch regression in animal models (8). On a molecular level, there are a variety of cardiac specific transcription factors which are integral to the regulation of heart development. They initiate heart development, determine cardiac cell fates, control expression of contractile proteins, and control the morphological development of heart structures (17). Mutations in the genes encoding these transcription factors have the potential to cause defective cardiac development.

Three of the most important transcription factors identified to date which are involved in heart development include *NKX2.5*, *GATA4*, and *TBX5*. *WNT* and *BMP* provide the first signals to initiate cardiac development; these signals target *NKX2.5*. *NKX2.5* is a homeobox gene which is expressed in cardiac progenitor cells and involved in the development of the heart tube and left ventricle, which originate from the first heart field. *GATA* transcription factors regulate cardiac contractile proteins and other regulatory genes, including *NKX2.5*, *MEF2*, and *HAND*. *MEF2* is involved in the differentiation of types of muscles in the heart, activating certain contractile proteins, and the development of structures from the second heart field. *HAND* genes regulate growth of the ventricles. *TBX* genes control cardiac fate, differentiation and the development of

the dorsal vessel, and have been associated with the development of the atria, ventricles, and the cardiac conduction system (17).

Even though a heart defects may originate with a single defective step of cardiac development, cardiac defects often evolve in a progressive manner throughout gestation, even after the completion of heart development at day 49. For example, hypoplastic left heart syndrome (HLHS) is thought to originate with changes in cardiac anatomy which alter the function of left ventricular inflow or outflow, but progression of the heart defect into the complete entity of HLHS occurs throughout gestation (8).

Although there have been many advances in our understanding of the molecular and developmental mechanisms which result in CHD, our understanding is not yet sufficient to classify types of CHD based on their developmental origin. Therefore, CHD can be categorized on the basis of clinical features, such as cyanotic versus non-cyanotic defects, or the anatomical segment of the heart that is affected. CHD may also be classified on the basis of complexity, as each individual with CHD may have only one isolated defect or a combination of multiple defects. A classification strategy developed by Botto et al., based on cardiac anatomy, was utilized to categorize types of CHD for the purposes of this study. Cardiac phenotypes are categorized into Level 1, 2, and 3 groups, from detailed to broad categories, respectively (18). Broad categories of CHD (Level 3 groups) are listed as the section headings below, which are further subdivided into Level 2 groups. Although the etiology of all types of CHD is largely unknown, most have been associated with various genetic syndromes. A description of each type of CHD and the most common associated genetic syndromes are provided below, but the genetic causes are discussed in greater detail in Section 1.1.3.2. A diagram of normal heart anatomy is provided below for comparison with each description of defective heart structure.

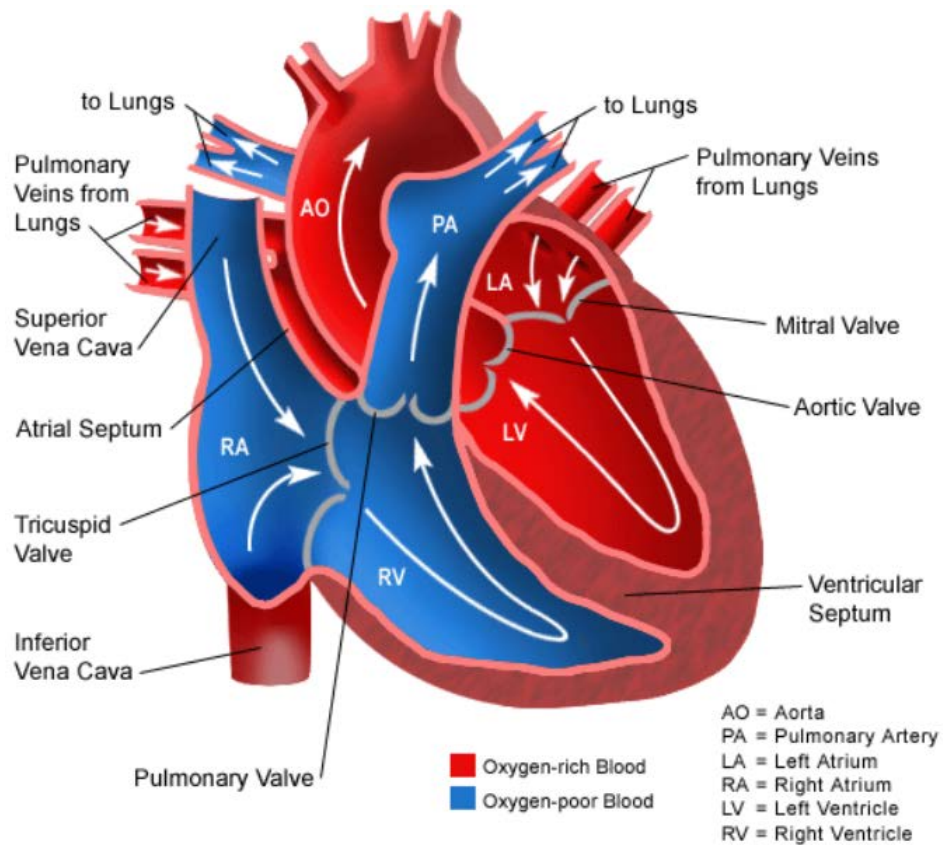


Figure 2. Normal heart anatomy (19)

1.1.2.1 Outflow tract defects

Outflow tract defects limit either the flow of oxygen-rich blood through the aorta or oxygen-poor blood through the pulmonary artery, which can result in hypertension or ventricular hypertrophy. Right ventricle outflow tract obstruction defects (RVOTO), which are obstructions of the pulmonary outflow tract, include the following:

- Pulmonary atresia (PA): Absence of the pulmonary valve; when the ventricular septum is intact, a patent ductus arteriosus provides the only pulmonary blood flow (20).

When associated with a ventricular septal defect, it is a variant form of tetralogy of Fallot.

- Ebstein's anomaly: Downward displacement of an abnormally structured tricuspid valve into the right ventricle, which it is often associated with functional pulmonary stenosis or atresia (20). The majority of cases are sporadic, but it can be associated with maternal lithium exposure during the first trimester (4, 21).
- Pulmonary valve stenosis (PS): Narrowing of the pulmonary valve which restricts blood flow from the right ventricle to the pulmonary artery. PS accounts for 7-10% of all CHD (20), and is very common among patients with Noonan syndrome, Alagille syndrome, and a variety of other genetic syndromes and chromosome abnormalities (4). In approximately 50% of cases of PS due to valve dysplasia, a mutation in *PTPN11*, associated with Noonan syndrome, is detected (20).
 - Peripheral pulmonary stenosis is a distinct entity caused by constrictions of the pulmonary artery; this can be associated with both Williams and Alagille syndromes (20).
- Tricuspid atresia: Absence of the tricuspid valve between the right atrium and right ventricle, causing the systemic venous return to enter the left heart through a patent foramen ovale or ASD (20). The majority of cases are sporadic (4).

Left ventricle outflow tract obstruction defects (LVOTO) are obstructions of the aortic outflow tract which include the following:

- Aortic valve stenosis (AS): Narrowing of the aortic valve between the left ventricle and the aorta; this is frequently associated with other types of CHD including mitral stenosis and coarctation of the aorta (20). It can be associated with a variety of chromosome abnormalities, Noonan syndrome, and Turner syndrome (4).

- Aortic atresia: Absence of the aortic valve which connects the left ventricle and the aorta (20). This defect has been associated with 11q deletion, Turner syndrome, Trisomies 13 and 18, and 4p deletion (4).
- Supravalvular aortic stenosis (SVAS): Narrowing of the aorta, commonly associated with Williams-Beuren syndrome (4).
- Interrupted aortic arch type A (IAA, A): Blockage between the ascending and descending aorta, just beyond the branch leading to the subclavian artery; this defect results from abnormal aortic arch development, between weeks 5 and 7 of gestation (22).
- Coarctation of the aorta (COA): Narrowing of the aorta which primarily occur at the origin of the ductus arteriosus (20). This defect is frequently seen in Turner syndrome, but has also been associated with other chromosome abnormalities (4). When associated with mitral valve abnormalities and AS, it is referred to as the Shone complex (20).
- Hypoplastic left heart syndrome (HLHS): This is a group of related defects, including underdevelopment of the left heart (left ventricle, aortic valve, and mitral valve) and of the ascending aorta (20). For blood to flow into the aorta, it must flow through an atrial septal defect into the pulmonary artery, and then through a patent ductus arteriosus. HLHS can be associated with various chromosome abnormalities, such as 11q deletion, Turner syndrome, Trisomy 13 and 18, and 4p deletion; and also in familial aggregation of left-sided obstructive heart defects (4).
- Bicuspid aortic valve (BAV): Aortic valves are typically tricuspid in structure, but up to 2% of the general population have a bicuspid aortic valve (7). Because of its high prevalence, BAV will not be reported in this study.

1.1.2.2 Septal defects

Septal defects are openings in the septum dividing the left and right atria or ventricles, allowing the oxygen-rich blood in the left heart to mix with oxygen-poor blood in the right heart. These defects may be associated with left outflow tract obstruction defects, such as coarctation of the aorta or aortic stenosis (4, 18). Septal defects include the following:

- **Ventricular septal defects (VSD):** A VSD is an opening in the septum between the ventricles, which allows oxygen-rich blood to flow into the right ventricle. VSDs are the most common type of CHD, accounting for 30-35% of all major heart defects. VSDs may be further categorized depending on their location in the ventricular septum; membranous VSDs are most common (20). VSDs are a common finding in Holt-Oram syndrome, familial ASD, and various other genetic syndromes (4).
- **Atrial septal defects (ASD):** An ASD is an opening between the atria which allows oxygen-rich blood to flow into the right atrium instead of the left ventricle. ASDs, along with PDA, are the second most common type of heart defect, accounting for 6 to 8% of all heart defects (20). Depending on location in the atrial septum, ASDs may be further defined as secundum type, sinus venosus, or coronary sinus (18). The majority of ASDs are sporadic, but may be associated with Holt-Oram syndrome, familial ASD, Ellis-van Creveld syndrome, and various other genetic syndromes (20).

1.1.2.3 Atrioventricular Septal Defects

Atrioventricular septal defects (AVSDs) are also referred to as endocardial cushion defects, because the endocardial cushions are the developmental precursors of the septum and the mitral and tricuspid valves (23). AVSDs typically includes contiguous atrial and ventricular septal

defects along with abnormalities of the atrioventricular valves (mitral and tricuspid) (20).

Approximately 60% of infants with AVSD have Down syndrome, but AVSDs may also be associated with other chromosome abnormalities (mainly deletion 8p and deletion 3p) and various monogenic syndromes (4, 24). Types of AVSDs include:

- Complete atrioventricular septal defects (AVSD): An AVSD is a large opening in the center of the heart which allows blood flow between all four heart chambers; this is associated with one common atrioventricular valve in place of the mitral and tricuspid valves. This is the most common form of AVSD among individuals with Down syndrome (20).
- Transitional AVSD: Transitional AVSDs involve only the atrial or ventricular portion of an AVSD; there are often two separate atrioventricular valves (20, 24):
 - Inlet-type VSD: The isolated ventricular component of an AVSD, which is an opening in the posterior ventricular septum beneath the tricuspid valve (20)
 - Primum type ASD: The isolated atrial component of an AVSD, which is an opening in the region of the mitral and tricuspid valves (20).

1.1.2.4 Conotruncal heart defects

Conotruncal defects arise from abnormal development during septation of the aortic and pulmonary outflow tract (14). As a group, conotruncal defects account for a large portion (25-30%) of all apparently isolated CHD (25). DiGeorge syndrome has been identified as a cause for a significant portion (6%) of all cases of conotruncal defects (13), but other chromosome abnormalities have been associated with this type of CHD as well (4). Conotruncal defects include:

- Tetralogy of Fallot (TOF): A combination of heart defects including a VSD, pulmonary stenosis, an aorta that overrides the ventricular septum, and hypertrophy of the right ventricle (20). TOF can be associated with various genetic syndromes, mainly including DiGeorge syndrome, Alagille syndrome, and Cat-eye syndrome (22pter→q11 duplication) (4).
- Double outlet right ventricle (DORV): A defect in which both the pulmonary artery and aorta stem from the left ventricle. This can be associated with additional defects; for example, Taussig-Bing malformation includes DORV, SVD, aortic stenosis, and possibly COA (20). DORV has been associated with various chromosome abnormalities and trisomies, but rarely is associated with DiGeorge syndrome (4).
- Persistent truncus arteriosus: The pulmonary artery and aorta are replaced by a single blood vessel (truncus arteriosus) which stems from the right and left ventricles; this must be associated with a VSD (20).
- Interruption of the aortic arch (IAA, types B and C): IAA, Type B is a blockage between the left carotid artery and left subclavian artery; it is the most common type of interrupted aortic arch defects. IAA type C is a blockage between the innominate artery and left carotid artery (22). Both IAA and truncus arteriosus have been associated with DiGeorge syndrome, Trisomy 8, and deletion 10p (4).
- Conoventricular VSD: An opening at the location where two portions of the ventricular septum meet below the pulmonary and aortic valves (26).
- Transposition of the great arteries (D-TGA): An inversion of the pulmonary artery and aorta, resulting in the aorta originating from the right ventricle and the pulmonary artery originating from the left ventricle, while the systemic and pulmonary veins return

normally (20); it may or may not be associated with a ventricular septal defect. It has been associated with various chromosome abnormalities, Trisomy 21, and Trisomy 18, but rarely is associated with DiGeorge syndrome (4).

1.1.2.5 Anomalous-Pulmonary Venous Return

Anomalous-Pulmonary Venous Return (APVR) defects cause drainage of one or more pulmonary veins into the systemic circulation system (20). APVR defects may be isolated or associated with outflow tract obstruction defects (18). Types of APVR include:

- Total anomalous-pulmonary venous return (TAPVR): All of the pulmonary veins drain into the right atrium instead of the left atrium, allowing all oxygenated and deoxygenated blood to mix; the presence of an ASD or PFO is necessary to maintain any systemic blood flow (20). The majority of cases are sporadic(4).
- Partial anomalous-pulmonary venous return (PAPVR): One or several pulmonary veins drain into the right atrium instead of the left atrium; this is frequently but not always associated with an ASD (20).

1.1.2.6 Patent Ductus Arteriosus

Patent Ductus Arteriosus (PDA) is an opening between the pulmonary artery and aorta which fails to close properly at birth, resulting in shunting of blood from the aorta to the pulmonary artery. This can increase strain on the heart and result in pulmonary hypertension, depending on the size of the PDA (20). Because the ductus arteriosus closes at birth, PDA is a very common finding among premature infants and it accounts for 6-8% of all heart defects (7). It is also present in 10% of patients with other types of CHD as is sometimes necessary to maintain

viability (20). For this reason, PDA will not be reported in our study if it is combined with other structural defects.

1.1.2.7 Laterality defects

Laterality defects have not been associated with any well-defined genetic syndromes, but have been reported in patients with chromosome abnormalities. This category can be subdivided into the following (4):

- Heterotaxy: Abnormal placement of organs, including the heart, on the opposite side of the body. This may be isolated or associated with other defects in cardiac structure, including ASD, VSD, AVSD, ventricular hypoplasia, pulmonary stenosis or atresia, or APVR defects (18, 20).
 - Asplenia: Both atria have the structure of the right atrium; this is also referred to as right atrial isomerism; this is associated with a central liver, absent spleen, and two structurally right lungs (20).
 - Polysplenia: Both atria have the structure of the left atrium; this is also referred to as left atrial isomerism; this condition is associated with several small spleens, absence of part of the vena cava, and two structurally left lungs (20).

1.1.2.8 Complex and Single Ventricle Defects

The following types of heart defects are included in this category:

- Complex cardiac malformations: A combination of heart defects including three or more defects (excluding simple ASD or VSD) (18).

- Single ventricle heart defects (SV): Single ventricle defects include double inlet left ventricle (DILV), in which only the left ventricle is developed, and double inlet right ventricle (DIRV), in which only the right ventricle is developed(18); both allow total mixing of systemic and pulmonary blood flow. These may be associated with transposition of the great arteries (20).
- L-transposition of the great arteries (L-TGA): Transposition of the aorta and pulmonary artery, as well as reversal of the right and left ventricle, resulting in the complete reversal of normal blood flow. When isolated, this defect has a better prognosis than D-TGA, however it is often associated with additional defects (18, 20).

1.1.3 Etiology and Recurrence of CHD

Based on early epidemiologic studies, approximately one quarter of CHD has an identifiable chromosomal or environmental cause, but the majority of cases of CHD have no identifiable cause. Among CHD with no identifiable cause, the minority of cases are associated with extracardiac features (syndromic CHD) but the majority of cases have no apparent extracardiac manifestations (isolated CHD). In cases in which the etiology is unknown, it may be difficult to determine the accurate recurrence risk. Based on early epidemiologic studies, the general recurrence risk for CHD has been estimated as 2 to 5% (27).

Various studies have attempted to characterize recurrence risk estimates for specific types of CHD. In a recent study based on the Danish national population and health registers, the relative risk for a first degree relative to have the same type of CHD was in the range of 2- to 6-fold, depending on the type of CHD (RR= 1.8- 5.2), while the relative risk for a first degree

relative to have a discordant type of CHD was still nearly 3-fold (RR= 2.68) (21). In a continuation of this study, it was hypothesized that shared gene mutations or epigenetic factors might increase susceptibility for all types of CHD which originate from the same embryologic segment. This could cause an increased relative risk for specific pairings of CHD within a family. However, results of this study showed that all combinations of discordant CHDs had very similar relative risks, in the range of 2- to 4- fold (22).

These data regarding recurrence risk have led to various hypotheses regarding genetic mechanisms which could contribute to CHD with an unidentified cause. Modes of inheritance which are consistent with a recurrence risk in the range of 2 to 5% include multifactorial inheritance or polygenic inheritance (8, 27). Multifactorial inheritance describes traits caused by complex interactions between many genes and environmental factors. Polygenic inheritance describes traits caused by interactions between multiple genes. The aggregation of dissimilar types of CHD in the same family could be attributed to pleiotropy, defined as multiple phenotypic effects caused by variation in the same gene (28). The similarity in recurrence risk between all combinations of discordant CHD would be consistent with various genetic mechanisms, including syndromes associated with multiple types of CHD, allelic variants within the same gene which cause distinct types of CHD, and shared environmental factors which can impact different stages of cardiac development and lead to different types of CHD (29).

According to the hypotheses detailed above, it is generally thought that many cases of CHD without an identifiable cause result from the combination of multiple genetic alterations which increase susceptibility to CHD along with the interaction of environmental risk factors (4). However, it has recently been suggested that the relatively low recurrence risks would also be consistent with a high de novo rate of pathogenic mutations or submicroscopic chromosome

abnormalities (30). In addition, the similar risks for concordant and discordant CHD within the same family could also be consistent with reduced penetrance and variable expressivity, which are characteristic of submicroscopic chromosome abnormalities. These genomic imbalances are recently being studied in the context of CHD and may prove to be an important contributor to the etiology of CHD with an unidentified cause.

1.1.3.1 Environmental Factors

The most common environmental factors which can affect cardiac development and cause CHD include maternal disease and teratogenic exposures during pregnancy. Maternal diseases which increase risk for CHD include rubella infection, phenylketonuria, and diabetes. For example, pre-gestational diabetes causes a relative risk in the range of 3-7 (29), which may be slightly higher than relative risk of having a first degree relative with CHD. Maternal teratogenic exposures which increase risk for CHD include retinoic acid, lithium, antiepileptic drugs including valproic acid and phenytoin, and alcohol (20, 30). Paternal exposures may also play a role, as pre-conception paternal occupational exposure to chemicals has recently been associated with increased risk for CHD in the fetus (31).

1.1.3.2 Genetic Factors

There are many genetic factors which have been associated with congenital heart defects, including single-gene defects, large chromosome abnormalities, and submicroscopic chromosome abnormalities. Single gene mutations have been associated with both isolated CHD and syndromic CHD, while large chromosome abnormalities are generally associated with syndromic CHD. Submicroscopic chromosome abnormalities have been implicated as a cause

for both isolated and syndromic CHD, although the reported prevalence and clinical significance of submicroscopic chromosome abnormalities among patients with CHD have been variable.

Many single genes associated with CHD have been discovered thus far, but it is thought to be unlikely that single gene defects account for the majority of isolated CHD, as the majority are likely to be due to multifactorial inheritance (4). Mutations in single genes which have been reported as a cause of isolated CHD include *NKX2.5*, *CFC1*, *PROSIT240*, *ZFPM2/FOG2*, *CRELD1*, *TUPLE1*, *GATA4*, *ZIC3*, *ACVR2B*, *LEFTY1*, and *NOTCH1* (4, 8). Several of these genes encode cardiac specific transcription factors which are described in detail in Section 1.1.2.

Three of the most well-known monogenic syndromes associated with CHD include Holt-Oram syndrome, Noonan syndrome, and Alagille syndrome. The majority of patients diagnosed with Holt-Oram syndrome carry heterozygous mutations in *TBX5*, a key transcription factor that regulates gene expression during embryogenesis. Classic cardiac features of Holt-Oram are found in seventy-five percent of patients and include specific congenital heart defects (ASD and/or VSD) or progressive AV cardiac conduction disease. Holt-Oram syndrome is also characterized by upper-limb radial ray malformations (4, 8, 32). Noonan syndrome is characterized by short stature, characteristic facies, webbed neck, developmental delay (DD), and chest deformities among other features. Eighty to ninety percent of patients with Noonan syndrome have CHD; the most common cardiac features include hypertrophic cardiomyopathy, PS, COA, ASD, VSD, AVSD, and TOF (4, 33). Noonan syndrome is genetically heterogeneous; the majority of Noonan syndrome is caused by heterozygous mutations in *PTPN11*, *KRS*, or *SOS1* but other genes may exist which have not yet been discovered (4). Similarly, Alagille syndrome is most often caused by a heterozygous mutation in *JAG1* or *NOTCH2*, but can also be caused by a microdeletion of 20p12. The most common types of CHD associated with Alagille

syndrome are peripheral pulmonary artery stenosis, ASD, VSD, TOF, and COA (34). Other characteristic clinical features of Alagille syndrome are cholestasis, congenital heart defects, posterior embryotoxon in the eye, characteristic facies, and butterfly vertebrae (35).

Other single gene disorders associated with congenital heart defects include Char syndrome (*TFAP2B*), CHARGE association (*CHD7*), Ellis-van Creveld syndrome (*EVC*, *EVC2*), Marfan syndrome (*FBN1*), Marfan-like syndrome (*TFGBR2*), Cardiofaciocutaneous syndrome (*KRAS*, *BRAF*, *MEK1*, and *MEK2*), and Costello syndrome (*HRAS*). In addition, DiGeorge syndrome, is most often caused by a microdeletion of 22q11.2, may also be caused by mutations in *TBX1* which is located in the 22q11.2 region. The most common types of CHD associated with DiGeorge syndrome include conotruncal defects (TOF and truncus arteriosus), IAA, and VSD. Classic extracardiac features of DiGeorge syndrome include palate abnormalities, immune deficits, hypocalcaemia, and learning difficulties (8, 36).

Large chromosome abnormalities are another well-documented cause of syndromic congenital heart defects. Classical cytogenetic analysis has been clinically available since the development of high resolution banding methods in the 1970s (37). This type of chromosome analysis uses a G-banded karyotype to visualize chromosomes at metaphase in order to identify aneuploidies and large chromosomal aberrations or rearrangements. The estimate that chromosome abnormalities account for 8 to 13% of all CHD originated from early epidemiology studies from the late 1980s (3, 38). However, these studies have several limitations. They took place before the development of more advanced technologies for chromosome analysis, so the only chromosome abnormalities included were aneuploidies and large chromosomal rearrangements detectable by classical cytogenetic analysis (10). In addition, chromosomal analysis was often ordered only when extracardiac abnormalities indicative of a genetic

syndrome were present, so patients with apparently isolated CHD would likely not have received chromosome analysis.

The most common aneuploidies associated with congenital heart disease include Trisomy 21 (Down syndrome), Trisomy 18, Trisomy 13, 45,X (Turner syndrome), and 47,XXY (Klinefelter syndrome). Down syndrome is the most common chromosomal aneuploidy in the general population; among individuals with Down syndrome, the prevalence of heart disease is between 40 to 50%. In this population, AVSDs account for the majority of heart defects, but the remainder include VSD, ASD, TOF, and D-TGA. Trisomies 13 and 18 have a much lower incidence in the general population, but are associated with a much higher prevalence of CHD. Approximately 80% of infants with Trisomy 13 and 90-100% of infants with Trisomy 18 have CHD. Among individuals with Turner syndrome, 25-35% have CHD, including COA, BAV, valvar aortic stenosis, HLHS, and aortic dissection. Among individuals with Klinefelter syndrome, approximately 50% have CHD, including mitral valve prolapse, PDA, and ASD (4). Large chromosome abnormalities associated with CHD include Cri-du-chat syndrome (deletion 5p, critical region at 5p15), Wolf-Hirschhorn syndrome (deletion 4p, critical region at 4p16.3), deletion 8p syndrome (ranging from 8p21→8pter), deletion 10p (ranging from 10p13→10pter), and Jacobsen syndrome (deletion 11q23). The prevalence of congenital heart defects among individuals with these large chromosome abnormalities is high, often in the range of 30 to 75% of all individuals affected (4). For example, the prevalence of CHD among individuals with Cri-du-chat syndrome is approximately 30-60%, which primarily includes PDA, VSD, ASD, and TOF (4).

Fluorescence in situ hybridization (FISH) is a more recent technology which became clinically available in the late 1980s. The purpose of targeted FISH is to identify submicroscopic

chromosome abnormalities associated with microdeletion or microduplication syndromes; these chromosome abnormalities are often too small to be visualized by classical cytogenetic analysis. FISH uses fluorescent probes, with both test and control sequences, which hybridize to metaphase chromosomes. After hybridization, fluorescence microscopy is used to visualize the number of probes which have hybridized to the region of interest. In targeted FISH analysis, the presence of only one copy of the test probe indicates a deletion of the region of interest, two copies of the test probe indicate a normal sequence, and three copies of the test probe indicate a duplication.

Subtelomere FISH is a specialized form of FISH which can identify chromosome abnormalities in the gene-rich subtelomeric regions. These regions contain unique chromosome-specific sequences. Among individuals with clinical indications including multiple congenital anomalies (MCA), developmental delay/ intellectual disability (DD/ID), dysmorphic facies, and other specific congenital anomalies, the prevalence of subtelomeric chromosome abnormalities has been reported as 4 to 9%. Subtelomeric rearrangements have also been reported in children with congenital heart defects, including aortic arch abnormalities, VSD, ASD, MVI, and PS with VSD (39, 40). Due to such evidence, Schellberg et al. concluded that subtelomeric FISH has significant clinical utility as a diagnostic tool for individuals with MCA with mental retardation and/or CHD and an apparently normal karyotype (41).

Some of the most common submicroscopic rearrangements associated with CHD include Williams-Beuren syndrome (microdeletion 7q11.23), Smith-Magenis syndrome (microdeletion 17p11.2), and DiGeorge syndrome (microdeletion 22q11.2). Although these syndromes have a relatively low prevalence in the general population, the prevalence of CHD within these syndromes is very high. For example, approximately 53 to 85% of patients with Williams-

Beuren syndrome and 75% of patients with DiGeorge syndrome have a congenital heart defect (4). The association between DiGeorge syndrome and CHD has been well-studied. Among patients with DiGeorge syndrome, conotruncal defects including TOF, TOF with pulmonary atresia, truncus arteriosus, and IAA, are the most common type of CHD. However, almost all types of CHD have been reported in patients with DiGeorge syndrome; this exemplifies the phenotypic variability which is characteristic of submicroscopic genomic imbalance syndromes. The developmental basis of the CHD in DiGeorge syndrome has been associated with the haploinsufficiency of the genes *TBX1*, *CRKL*, and *ERK2*, which causes dysfunction of neural crest cells and the anterior heart field (42).

Targeted FISH can be used to test for any of these syndromes, but it is generally reserved for patients with clinical features which are indicative a specific genetic syndrome. Among neonates, this can present a challenge, as extracardiac features may be subtle or not yet apparent. Because of the high prevalence of CHD among patients with DiGeorge syndrome and the difficulty of recognizing additional features, FISH has been utilized by some as a first-tier test for neonates with CHD. Baker et al. analyzed the detection rate of FISH for 22q11.2 among 110 neonates and infants with CHD admitted to the Cardiac Intensive Care Unit at Children's Hospital of Pittsburgh. In this population, the addition of FISH for 22q11.2 did increase the number of diagnoses by 5% above the 14% detection rate of chromosome abnormalities by classical cytogenetic analysis alone, to yield a total detection rate of 19% (43).

1.1.4 Chromosomal Microarray Analysis and Copy Number Variants

A novel technology which is now widely used as a clinical diagnostic tool is chromosomal microarray (CMA). CMA is the equivalent of hundreds to thousands of FISH tests for microdeletions and microduplications (44); it detects submicroscopic chromosome abnormalities, also known as copy number variants (CNVs), across the entire genome at a high resolution. By definition, CNVs are any DNA segment greater than 1 kb in length with which have variable copy number in comparison with a reference genome (45). CNVs can occur anywhere in the genome, although they are most frequent in regions flanked by low copy repeats (LCRs), which are sequences with 98-99% sequence identity (44). They are most often too small to be detected by classical cytogenetic analysis. They may be benign or pathogenic, due to abnormal dosage or dysregulation of one or more genes in the region of loss or gain (44).

The term CMA includes microarray-based comparative genomic hybridization (array CGH) and single nucleotide polymorphism (SNP) arrays; array CGH is the type of chromosomal microarray utilized in our study. This test can be performed by using cloned bacterial artificial chromosomes (BACs) or synthesized DNA fragments (oligos). These fragments are designed to match specific loci across the entire genome and are fixed to a glass surface. Patient and control DNA is labeled and then allowed to hybridize to the DNA fragments on the glass microarray chip. The intensity of the hybridization patterns is analyzed to detect any variation copy number between patient and control DNA.

Over time, the coverage and resolution of array platforms has evolved. Targeted BAC arrays were the first to be developed. These included hundreds of BAC clones which were targeted to specific regions in the genome with known clinical significance, such as

microdeletion syndrome regions, subtelomeric regions, and pericentromeric regions. Targeted arrays simultaneously test regions with known clinical significance. Their interpretation is uncomplicated, but they are not able to detect potentially pathogenic CNVs which are located in regions without probe coverage. Whole genome arrays were developed next, with BAC clones covering the backbone of the entire genome. Oligonucleotide arrays are the most recent form of whole genome arrays, which have a higher density of probe coverage across the genome. Current oligonucleotide arrays generally have a resolution of 20-50 kb in targeted regions of the genome and 100-250 kb in backbone regions. The majority have a minimum resolution of at least 400 kb across the genome (16). Overall, the resolution of array CGH is at least five- to ten-fold greater than classical cytogenetic analysis, which is generally limited to the detection of chromosome aberrations greater than 5 to 10 Mb (12, 16, 45).

Many advantages exist for the use of array CGH in comparison with standard cytogenetics. Due to its higher resolution, array CGH is able to determine with accuracy which genes are included in the region of copy number variation and may be able to identify specific genes which are disrupted by the breakpoints. Even if a copy number variant is large enough to be detected by standard cytogenetic methods, accurate identification of the genes involved is often not possible without the use of array CGH (46). Identifying the genes which may have altered expression can provide very important prognostic information and aid in the interpretation of results. Studies have shown that the diagnostic yield of array CGH is significantly higher than that of classical cytogenetic analysis among individuals with various clinical indications, including MCA, autism spectrum disorders, and DD/ID. Additionally, the cost of array CGH is often less than the combination of classical cytogenetic analysis and a targeted FISH test (16).

Despite the many advantages, several limitations of array CGH must be considered when used in a clinical setting. Array CGH generally has a longer turn-around time than classical cytogenetic analysis. Some platforms may miss certain aneuploidies (such as XYY), marker chromosomes, and triploidies. Array CGH is not able to detect the majority of balanced rearrangements that would be detectable by standard cytogenetics, but in 20% of balanced rearrangements there is a loss or gain of material which would be detectable by array CGH (46). It may identify mosaicism in some cases, but only at a 10% level for aneuploidies and at a 20-30% level for other chromosomal abnormalities such as deletions and duplications (47); in comparison, standard cytogenetics is more accurate in the detection of mosaicism. Array CGH cannot detect point mutations responsible for Mendelian disorders. When a common aneuploidy syndrome is suspected due to the clinical presentation, array CGH may not be indicated as a first-tier test due to cost-effectiveness. It is also not indicated when there is suspicion for a balanced translocation due to a history of miscarriages, as it cannot detect any truly balanced rearrangements (46). The clinical acumen of the health care professional is highly valuable in determining the appropriate test to order, and in some cases classical cytogenetic analysis may be more efficient, cost-effective, and accurate.

One important ethical consideration associated with array CGH is the potential to detect incidental findings which are clinically relevant but unrelated to the original diagnosis (46). This may include detection of adult-onset disorders. Adult-onset genetic disorders are defined as disorders which are usually phenotypically asymptomatic until the third decade of life or later. It is the position of the National Society of Genetic Counselors (NSGC) that when the identification of gene carriers does not provide an avenue for therapeutic or preventative treatment in the prenatal or childhood periods, genetic testing must be carefully considered and

this decision should include genetic education and counseling (48). For instance, array CGH has the potential to identify copy number losses containing tumor-suppressor genes which are associated with adult-onset cancer predisposition disorders. This information would have important implications for medical management of the patient in the future but may only raise parental distress and anxiety in the present. Even the incidental detection of a severe disorder with childhood onset, such as Duchenne muscular dystrophy, may not be information that is desired by the parents. These findings are reported when they are detected, as the information may be useful for planning medical management and in some cases life-saving, but it raises a very important ethical consideration when ordering array CGH on a clinical basis.

Another challenge presented by the use of array CGH on a clinical basis is the interpretation of results. Various studies have estimated that CNVs account for 5 to 12% of the total genome (49, 50) and that the average person has 800 or more benign copy number variants (46). Therefore, CNVs may be classified as benign, pathogenic, or a variant of uncertain significance (VUS). Because whole-genome oligonucleotide arrays are able to detect CNVs throughout the entire genome, a larger proportion of the CNVs detected will have unclear clinical significance (16). In addition, providing prognostic information and recurrence risk based on results of array CGH may be challenging even when a CNV is clearly pathogenic. This can be due to the limited availability of previous case reports or the incomplete penetrance and variable expressivity associated with a particular CNV.

Determining an optimal resolution of an array CGH platform is critical in addressing the challenge of result interpretation. Lower resolution platforms may miss smaller pathogenic abnormalities, while higher resolution platforms may increase detection of VUS. Breckpot et al. compared 1 Mb and 244 kb array CGH platforms among patients with apparently syndromic

CHD, and concluded that causal CNVs were typically large and detected by a 1 Mb platform, while the 244 kb platform yielded a low frequency of causal variants and a high frequency of VUS (45). We used a 135 kb platform in this study, but CNVs less than 300 kb with no genes or only genes with no known clinical significance are not reported by the laboratory, in order to limit the number of variants of unclear significance detected.

Characteristics of CNVs which are suggestive of pathogenic versus benign status are outlined below (16). A summary of these criteria in algorithm form is shown in Figure 3 (45). This algorithm was utilized, in addition to the interpretation provided by the laboratory, to assess pathogenicity of all CNVs detected in our study.

Characteristics suggestive of pathogenic status:

- Identification of a similar CNV in an affected parent or relatives.
- Overlap with a region of genomic imbalance identified in a database of affected individuals.
- Overlap with a region associated with a known microdeletion or microduplication syndrome.
- CNVs containing OMIM genes associated with disease, specifically genes associated with CHD or dominant, monogenic diseases with a known dosage effect (12).
- Location in a gene-rich region. One proposed threshold is 20 genes, as the more genes are located in the region, the greater the probability that one is dosage-sensitive (12, 44).
- Large size: In a study by Thienpont et al., the median size of CNVs not previously reported as normal variants was approximately 6 Mb (12).
- Deletions, homozygous deletions, and amplifications greater than one copy.

- De novo origin: CNVs which are not detected by parental studies may be more likely pathogenic. However, this may not be a strong indicator of pathogenicity for small CNVs, as the frequency of small de novo CNVs in normal individuals is not known (12).

Characteristics suggestive of benign status:

- CNVs identified in a healthy parent or relative.
- CNVs within a region of genomic imbalance identified in a database of healthy individuals.
- Location in a gene-poor region.
- Duplications containing no regulatory elements and no genes which are known to be dose-sensitive.
- Small size: In one study, the median size of CNVs detected in normal individuals fell between 0.15 and 1.35 Mb (12).

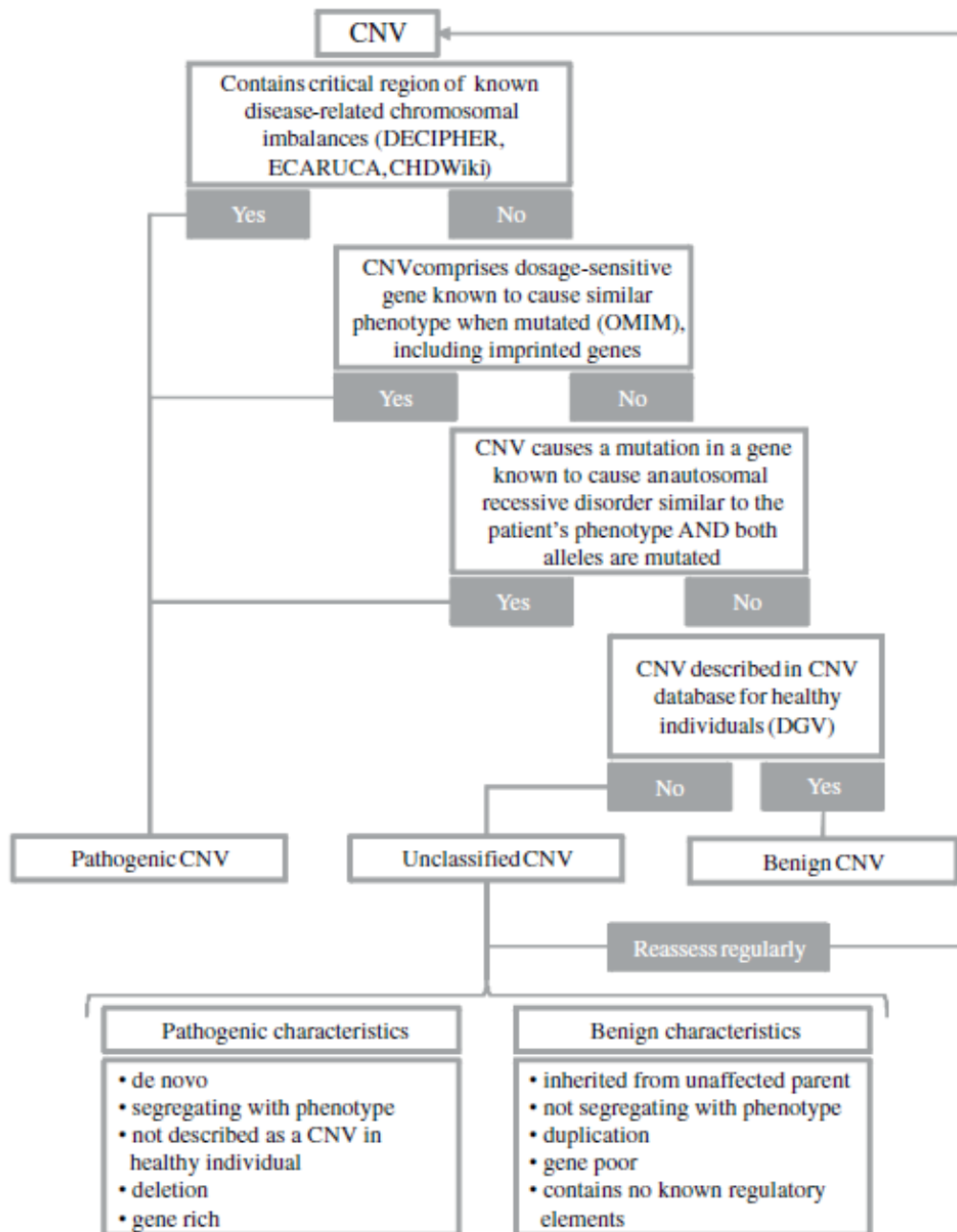


Figure 3. Assessment of Pathogenicity of Copy Number Variants (CNVs) (45)

The phenotypic effects of a CNV are largely dependent on the dosage sensitivity of genes in the region of loss or gain. Some genes are insensitive to dosage, and the deletion of one allele may have no effect. Gene associated with autosomal recessive metabolic syndromes are one example; the deletion is only pathogenic if there is little to no protein produced. Other genes are

highly sensitive to dosage, and the haploinsufficiency of these genes resulting from a deletion may cause a severe phenotype. Although duplications are not as likely to be pathogenic, the increased dosage of certain genes can also have phenotypic effects. Beaudet et al. postulated that many genes likely have intermediate sensitivity, allowing the phenotype to be modified by both genetics and environmental factors. This hypothesis would be consistent with the incomplete penetrance and variable expressivity which are common to disorders associated with copy number variation (44).

1.1.5 Array CGH and DD/ID, MCA, and ASD

Array CGH is currently widely used as a diagnostic tool among patients with developmental delay/ intellectual disability (DD/ID), multiple congenital anomalies (MCA), and/or autism spectrum disorders (ASD). Many studies have looked at the diagnostic yield of array CGH among patients with these clinical indications. A summary of several important studies performed to date is provided below, including the inclusion criteria, array CGH platform, and detection rates of CNVs reported in each study.

Lu et al. analyzed the results of array CGH among over 600 neonates with multiple congenital anomalies. Because this study only included neonates, clinical features such as autism spectrum disorders or DD/ID were not yet identifiable. For each subject included, one of three microarray platforms were utilized, including a V5 BAC-array, V6 BAC-array, and a V6-oligonucleotide array; which had targeted genomic coverage with increasing resolution, respectively. The detection rate was 27.1% among patients with dysmorphic features, MCA, and other clinical indications; 24.6% among patients with dysmorphic features with or without other

clinical indications, and 17.9% among patients with only MCA with or without other clinical indications(15).

Shaffer et al. used a BAC array with 622 loci, targeted to known microdeletion syndromes, subtelomeric regions, and pericentromeric regions plus some backbone probe coverage, to test a broad population of over 8,000 individuals with indications including DD/ID, seizures, and various congenital anomalies. They detected CNVs in 12% of this population, but after further assessment of pathogenicity of each CNV, only approximately 7% of this population was determined to have clinically significant chromosome abnormalities, 1% had benign polymorphisms, and 4% had variants of unclear clinical significance (51). Wincent et al. tested 160 patients with DD and/or MCA using array platforms with greater resolution, and identified CNVs which were not known to be normal variants in 22.5% of all patients. A total of 13.1% of all CNVs identified were presumably causal to the patient's phenotype. A portion of their study population was tested using a 38K BAC-array and the remainder was tested with a 244K oligonucleotide-array (52).

Miller et al. performed a meta-analysis of data which combined the findings of various studies including, in total, over twenty thousand patients with DD/ID, MCA and ASD. Among these patients, array CGH had a diagnosis rate ranging from 15 to 20%, compared to a diagnostic yield of approximately 3% by using classical cytogenetic analysis alone (excluding Down syndrome and other recognizable chromosomal syndromes) (16). In a similar study, Sagoo et al. also performed a meta-analysis of data including nearly 14,000 patients with learning disability and congenital anomalies. They reported a lower diagnostic yield of 10%, and a false-positive rate of 7% (53). It is clear from these studies that array CGH does result in an increased detection rate of chromosome abnormalities among patients with specific clinical indications.

However, it can also create a clinical challenge due to the detection of copy number variants which are benign or have unclear clinical significance.

Based on the evidence delineated above for the increased diagnostic yield of array CGH, the American College of Medical Genetics (ACMG) has recently recommended in their Practice Guidelines that array CGH be ordered as first-tier test for individuals with any of the following: MCA not specific to a well-delineated genetic syndrome, apparently non-syndromic DD/ID, or autism spectrum disorder. The goal of these recommendations is to increase the number of patients for which accurate and timely information about etiology, prognosis, and recurrence risks can be provided (46).

1.1.6 Array CGH and Congenital Heart Defects

Recently, various studies have investigated the clinical utility of array CGH among patients with CHD. These studies have provided valuable information on potential candidate genes for CHD and the importance of submicroscopic chromosome abnormalities as a contributing etiology of CHD. The benefit of utilizing array CGH in this population is that if a pathogenic and likely causal CNV is detected, additional prognostic information may be gained from previous case reports. In addition, should parental studies confirm that a CNV is inherited, the recurrence risk would be 50% (for an autosomal CNV), but variable expressivity must be considered. If the CNV is de novo, recurrence risk would be at the level of the general population (approximately 1%) rather than 2-5% (27). Several studies done to date regarding array CGH for patients with CHD are listed in Table 1 and summarized below; the primary

difference between these studies is the inclusion criteria, such as age or the presence or absence of additional syndromic features. Further research on this topic is warranted to further define the clinical utility of array CGH among specific populations of patients with CHD.

Although the majority of CHD is considered non-syndromic, 25-40% of all cases of CHD are associated with additional congenital anomalies (7). It has been hypothesized that submicroscopic chromosome abnormalities may be one underlying cause for these cases of apparently syndromic CHD without an identifiable cause. Thienpont et al. was the first to analyze the use of array CGH among patients with apparently syndromic CHD. They included 60 patients of unspecified ages with CHD and other syndromic features (a major malformation, mental retardation or special education, and/or three or more minor physical anomalies). These patients previously received a dysmorphology exam by a geneticist, classical cytogenetic analysis, and testing for specific genetic disorders if indicated and had no genetic diagnosis prior to array CGH. Among this population, the detection rate of chromosome abnormalities was 30% using a 1 Mb platform; however only 17% were considered to be causal for CHD (12). Breckpot et al. included the same study population, along with an additional 90 subjects with the same inclusion criteria, and reported a diagnosis rate of likely causal CNVs of 18% using a 1 Mb platform. The only feature which had significant predictive value for the detection of chromosome abnormalities was dysmorphism (45).

Richards et al. also tested 20 children with apparently syndromic CHD, in comparison with a matched control population of 20 children with apparently isolated CHD, all of whom had a normal karyotype. The detection rate of array CGH among children with CHD and any other congenital anomalies was 25%, but was nearly 50% among children with CHD and neurologic abnormalities (including developmental delay). In this study, no chromosome abnormalities

were detected among children with isolated CHD, which led the authors to suggest that children with CHD and neurologic abnormalities should receive subtelomeric FISH or microarray along with classical cytogenetic analysis (11). Goldmuntz et al. also looked at the detection rate of SNP-based oligonucleotide microarray among 58 patients of various ages with CHD and other congenital anomalies or dysmorphic features. Patients with a known genetic diagnosis or chromosome abnormality detectable by classical cytogenetic analysis were excluded. In this study population, the detection rate of potentially pathogenic CNVs was 20.7% (54).

In contrast with the previous studies, Erdogan et al. specifically analyzed the utility of array CGH among patients with apparently isolated CHD. Their study cohort included 105 Caucasian children diagnosed with an isolated CHD, who had no neurological problems or other features indicative of a syndrome at the time of diagnosis, and with the exception of one patient, had no family history of CHD. Among this population, 18 CNVs were detected which were not previously reported as normal variants, yielding a detection rate of 17% (30). As a subsection of a larger study including children with all types of birth defects, Lu et al. included 101 neonates with both apparently isolated and syndromic CHD, and reported a microarray detection rate of 21.8% (15).

Table 1. Summary of studies to date on array CGH for patients with CHD

| Study | Number of Subjects | Phenotypic Features | Total Detection Rate | Likely Causal Detection Rate |
|-----------------------------------|---------------------------------|----------------------------|--------------------------------|-------------------------------------|
| Thienpont et al. 2007 | 60 | Syndromic | 30% | 17% |
| Breckpot et al. 2010 | 60 (above) + 90 | Syndromic | 28.7% | 18% |
| Richards et al. 2008 | 20 | Syndromic | 25% | NA |
| Goldmuntz et al. 2011 (SNP-array) | 58 | Syndromic | 20.7% | NA |
| Goldmuntz et al. 2011 | Previous literature review | Syndromic | NA | 19% |
| Richards et al. 2008 | 20 | Isolated | 0% | NA |
| Erdogan et al. 2008 | 105 | Isolated | 17% | NA |
| Goldmuntz et al. 2011 | 40 + previous literature review | Isolated | NA | 3.6% |
| Lu et al. 2008 | 101 | Neonates with CHD | 21.8% (clinically significant) | NA |

There has been a wide range in the detection rates reported by previous studies, among both apparently isolated and syndromic CHD. Some studies have suggested that the detection rate of CNVs among isolated CHD may be comparable to the detection rate among syndromic CHD (30). However, there are often significant differences between the total detection rate by array CGH and the detection rate of likely causal CNVs. To clarify the discrepancies, Breckpot et al. overviewed the literature on this topic and reclassified the causality of all chromosome aberrations detected by array CGH. They reported a stark difference in the frequency of causal CNVs in each population, with a frequency of 3.6% among patients with apparently isolated CHD and 19% among patients with apparently syndromic CHD (45). The collective data

provided by these studies suggest a significant contribution of submicroscopic chromosome abnormalities to the etiology of CHD. Although the contribution appears to greatest among populations with apparently syndromic CHD, the contribution among populations with apparently isolated CHD is not insignificant.

1.2 SIGNIFICANCE

Array CGH has recently been added as a first-tier test, along with classical cytogenetic analysis, at our institution for any neonate with CHD admitted to the Cardiac Intensive Care Unit (CICU). This standard of care has been implemented on the basis of the recent ACMG recommendation to use array CGH as a first-line test for patients with multiple congenital anomalies, the documented increase in detection rate using array CGH for patients with CHD, and the known clinical benefit of obtaining an early diagnosis if an underlying genetic etiology exists. The implementation of this standard of care provides a unique opportunity to assess the detection rate and clinical significance of array CGH results in this population.

Previous studies have analyzed the clinical utility of array CGH among children and adults with CHD, but information regarding the detection rate and clinical efficacy of array CGH among neonates with CHD and as a first-tier test is still limited. In studies which have included children and adults with CHD, extracardiac features are more readily apparent and in some cases, testing by array CGH was only performed after other genetic diagnoses were ruled out. Our study contributes valuable information due to our unique study population, which includes any neonates with CHD admitted to the CICU within the first four weeks of life. Due to presentation

in the neonatal period, this population may also have a higher prevalence of complex and clinically severe heart defects in comparison with previous studies.

Compared to previous studies, we hypothesize a lower detection rate than the detection rate among populations selected for features that would not be apparent during the neonatal period, such as neurologic abnormalities (11). Lu et al. included a very similar population of neonates with both isolated and syndromic CHD, so we hypothesize that our detection rate of CNVs will be similar to their detection rate of 21.8%. Their study was a large, epidemiologic study which included neonates with all types of birth defects, so they understandably do not provide information regarding the prevalence of syndromic versus apparently isolated CHD in their population, and they report minimal information about specific CNVs (15). Our study will supplement this previous data by including additional information regarding the presence of syndromic features among neonates with a CNV, as well as an assessment of the potential causality of each CNV detected.

It has been clear from previous studies that the frequency of causal CNVs is much higher in populations with apparently syndromic CHD (45), so it could be argued that chromosomal studies including array CGH should only be done for individuals with CHD and extracardiac abnormalities. However, not all extracardiac abnormalities or dysmorphic features will become apparent during the neonatal period. Features such as developmental delay and cognitive deficits only become apparent during childhood. Dysmorphic features may be present at birth, but they may be very subtle and difficult to detect during the neonatal period. Even after the neonatal period, some dysmorphic features which are indicative of a genetic syndrome may only be readily detected by a geneticist specializing in dysmorphology. In cases with an underlying genetic etiology, it can be problematic to wait to pursue genetic testing until additional

extracardiac features are apparent. Postponing a genetic diagnosis may also postpone effective management strategies and in some cases could have significant clinical consequences. Furthermore, it could also limit recurrence risk information available to parents when making reproductive decisions. For these reasons, it can be problematic to base genetic testing decisions on the presence of extracardiac features, especially among neonates. Our study will contribute valuable information to further clarify whether array CGH provides meaningful clinical information when ordered as a first-tier test for all neonates with CHD regardless of the presence of syndromic features.

1.3 RESEARCH QUESTIONS AND HYPOTHESES

The broad purpose of this study is to improve the clinical care of patients with a CHD, through increasing our knowledge of the genetics of CHD and determining the most efficient genetic testing strategy for this patient population. This study is designed to supplement the findings of previous literature regarding the contribution of submicroscopic chromosome aberrations to the etiology of CHD, and specifically to explore the clinical utility of array CGH as a first-tier test for neonates with CHD. Our study has both quantitative and qualitative aims which are discussed below.

1.3.1 Aim 1: Comparison of Detection Rates

The quantitative aim of this study is to compare classical cytogenetic analysis and array CGH for the purpose of diagnosing chromosome abnormalities in neonates with a CHD. The pertinent research question is the following: Does the use of array CGH increase the detection rate of chromosome abnormalities among neonates with CHD above that of classical cytogenetic analysis? To address this question, we will first determine whether the prevalence of chromosome abnormalities detected by classical cytogenetic analysis in our study population matches the reported literature rate at our institution. A previous study performed at the CICU of Children's Hospital of Pittsburgh of UPMC reported a detection rate of 14% among infants with CHD, using classical cytogenetic analysis and a total detection rate of 19% when including FISH for 22q11.2 (43). We will then determine whether the addition of array CGH increases the detection rate of chromosome abnormalities. We hypothesize that including array CGH as a first-tier test will significantly increase the detection rate of chromosome abnormalities in our study population. Specifically, we hypothesize that array CGH will increase the rate of detection of chromosome abnormalities by 6% when compared to the previously reported 14% rate of chromosome abnormalities detected by classical cytogenetic analysis among infants in the CICU at Children's Hospital of Pittsburgh.

1.3.2 Aim 2: Assessment of Clinical Significance of CNVs

One qualitative aim of this study is to assess the clinical significance of any CNVs detected by array CGH. Determining the proportion of CNVs in our population with known

clinical significance versus those with uncertain clinical significance is important to accurately assess the clinical utility of array CGH as a first-tier test in this population.

1.3.3 Aim 3: Analysis of Phenotypic Data

An additional qualitative aim of this study is to analyze the dysmorphology exam results for any associations between specific CHDs, chromosome abnormalities, and extracardiac abnormalities. We hypothesize that array CGH will be a useful first-tier diagnostic tool among an unselected population of neonates with CHD, including those with both apparently isolated and apparently syndromic CHD, as some patients with apparently isolated CHD may present with additional phenotypic features later in life. However, we would expect based on the findings of previous studies that the detection rate of array CGH will be the greatest among neonates with apparent syndromic features.

2.0 MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the University of Pittsburgh prior to initiating the study. Neonates under the age of four weeks diagnosed with CHD were ascertained through the CICU staff at Children's Hospital of Pittsburgh of UPMC. Enrollment began on April 4, 2011 and continued through March 16, 2012 for the purposes of this study; enrollment is still ongoing for additional statistical analysis in the future. Per standard of care, the majority of patients had classical cytogenetic analysis and array CGH ordered simultaneously by their attending physician, as well as a dysmorphology exam by a geneticist using a standardized form. This study involved a chart review and data analysis; no additional testing was performed for the purposes of this study. After obtaining consent from the patient's family for access to medical records, including genetic test results, the data outlined below was collected by a chart review of the patient's medical records and was compiled into a secure, password-protected database.

Results were obtained from any genetic tests ordered as standard of care, including classical cytogenetic analysis, array CGH, and FISH testing in some cases. With the inclusion of array CGH, FISH testing for 22q11.2 microdeletion syndrome is no longer indicated as a first-tier test for neonates admitted to the CICU at our institution, as genomic imbalances involving this region would also be detected by array CGH. However, this test was still ordered in some

cases, and the results are included in our analysis. Additional data obtained from medical records included gender, race, gestational age at birth, the first recorded height, weight, and head circumference percentiles, type of CHD, physical exam results, results of imaging studies (head ultrasound or MRI and renal ultrasound), and whether the patient had a formal genetics consult.

Regardless of receiving a formal genetics consult, the majority of patients included in our study received a baseline dysmorphology exam by a geneticist, per standard of care. The form used to evaluate for dysmorphology is included in the Appendix B. The general fields of this exam include head shape, facies, hair, eyes, ears, nose, mouth, tongue, neck, cardiac features, genitourinary, chest, back, lungs, diaphragm, hands, feet, joints, skin, nails, neurologic, and other significant clinical features. For each of these features, an assessment was made of normal, abnormal, or could not assess. Any descriptions of dysmorphic features were also included in our database. For patients who did not receive this standard dysmorphology exam by a geneticist, supplemental physical exam data were collected from Cardiology or CICU progress notes.

All genetic testing ordered by the CICU was performed at the Pittsburgh Cytogenetics Laboratory, which is CLIA-approved and housed at Magee-Womens Hospital of UPMC. To perform classical chromosome analysis, 5 mL peripheral blood was collected from the patient in a sodium heparin tube. High-resolution G-banding was used to visualize prometaphase chromosomes at a 675 band-level resolution. In each analysis, twenty cells were counted and six metaphase cells were analyzed and karyotyped.

To perform chromosomal microarray analysis, 5 ml peripheral blood was obtained in an EDTA tube. The microarray platform used by the Pittsburgh Cytogenetics Laboratory is the Roche NimbleGen 135K oligonucleotide array platform by SignatureChipOS™, version 2.0

(based on UCSC 2006 hg 18 assembly). The accuracy and precision of this test has been established and verified in accordance with CLIA '88 requirements. This platform analyzes 3397 loci at a 135 Kb resolution, and includes clones for subtelomeric regions, pericentromeric regions, and known genetic syndromes. Any copy number variants which are less than 300 Kb in size, which do not contain any genes or contain only genes with no known clinical significance, are not reported. Due to the limitations of microarray testing, this platform cannot detect balanced alterations, including reciprocal translocations, Robertsonian translocations, inversions, balanced insertions, or imbalances in regions which are not included on the platform. The accuracy of this specific platform for detecting mosaicism has not been established.

The demographics of our study population, including race, gender, prevalence of each type of CHD, and prevalence of isolated versus apparently syndromic CHD, are tabulated and reported in our results. Specific types of CHD were categorized by a standard classification system established by Botto et al. In addition, each case was categorized as a simple, association, or complex heart defect; this categorization may be relevant to a syndromic versus isolated etiology but does not necessarily correlate with clinical severity. Simple CHDs were defined as either an uncomplicated defect or a well-recognized unit of defects, such as TOF. Associations were defined as combinations of two to three simple defects. Complex CHDs were defined as combinations of three or more defects, not including VSD or ASD (18).

Categorization of isolated versus syndromic CHD was based on the presence of extracardiac features and dysmorphic features. Dysmorphic features were defined as any physical features which were noted as abnormal on a dysmorphology exam. Other features which were not noted to be abnormal were considered to be potentially normal variation; these are described in the case reports for patients with chromosome aberrations (Appendix A) but

otherwise were not reported. In many cases, an overall assessment of dysmorphic appearance versus normal appearance was made by a Geneticist following the physical exam; this was also utilized in categorizing subjects as dysmorphic or non-dysmorphic. Extracardiac abnormalities were defined by abnormal imaging studies or the presence of any congenital anomalies or additional diagnoses not related to the subject's heart defect. We did not include infections, features with a strongly suspected environmental cause, or additional cardiac abnormalities in this category. Patients were categorized as having apparently isolated CHD if they did not have dysmorphic features or extracardiac abnormalities. Patients were categorized as having apparently syndromic CHD if they had any dysmorphic features and/or extracardiac abnormalities.

Quantitative data analysis was performed to compare the detection rates of all types of genetic testing performed. For the purposes of this study, the total detection rate of array CGH will include both abnormal CNVs and variants of uncertain clinical significance (VUS). Statistical analysis was performed using a McNemar's test to compare the detection rates of classical cytogenetic analysis and array CGH. This analysis was done to address our primary aim of determining whether array CGH significantly increases the detection of chromosome abnormalities in our study population.

Qualitative data analysis of each CNV detected is presented in tabular fashion in Tables 3 and 4, and in case report fashion in Appendix A. The pertinent clinical features discussed in each case report include gestational age, growth parameters, extracardiac abnormalities, and dysmorphic features, and an assessment of the potential pathogenicity of the CNV. The following steps were taken to assess the pathogenicity of each CNV. Genoglyphix Genome Browser® and DECIPHER, v5.1 were utilized to identify any genomic imbalance syndromes

which overlap with the region of interest and any genes which are deleted or duplicated (55, 56). For any genes identified, OMIM was used to determine the dosage sensitivity and any genetic syndromes associated with that gene. The Database of Genomic Variants (DGV) was used to determine if the CNV is found in healthy individuals (57). If parental studies were performed, information regarding whether the chromosome abnormality was inherited or de novo was also included.

3.0 RESULTS

3.1.1 Demographics

The enrollment data and demographics of our study population are presented in Table 1. The gender ratio of our population was disproportionate in favor of males (62%) over females (38%). Our population also disproportionately represented Caucasian subjects, as 62% of our subjects were Caucasian, not including those with race not specified. Four patients (8%) were born prematurely at less than 37 weeks gestational age, although the vast majority of patients (92%) were greater than 37 weeks gestational age at delivery.

Table 2. Enrollment data and demographics

| | n | % of total |
|--------------------------|----------|-------------------|
| Subjects Enrolled | 50 | |
| Total Number Eligible | 59 | |
| Eligible, Not Enrolled | 9 /59 | 15.2% |
| Sex | | |
| Male | 31 | 62% |
| Female | 19 | 38% |
| Race | | |
| Caucasian | 31 | 62% |
| African American/ Black | 1 | 2% |
| Middle Eastern | 2 | 4% |
| Hispanic | 1 | 2% |
| NOS | 15 | 30% |
| Gestational Age | | |
| < 37 weeks | 4 | 8% |
| ≥ 37 weeks | 46 | 92% |

Figure 4 depicts the prevalence of simple, association, and complex CHDs within our study population. Based on our categorization criteria, defined in Section 1.4, the majority of subjects in this population (64%) had simple CHD. Twenty-six percent of the population had an association of two to three individual types of CHD, and 10% had complex CHD.

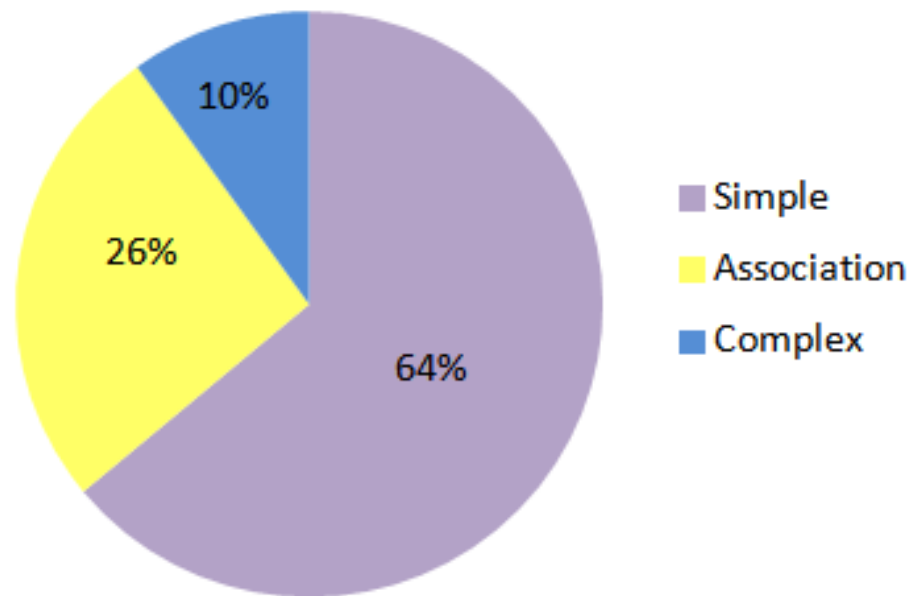


Figure 4. Complexity of CHD among all subjects enrolled

3.1.2 Detection Rates

The detection rates of each type of genetic test, including classical cytogenetic analysis, FISH for 22q11.2, and array CGH, are presented in Table 2. Each detection rate was calculated by dividing the number of abnormal results in Column 2 by the number tested in Column 3. The clinical significance and potential causality of each CNV are discussed in greater detail in Appendix A.

Table 3. Detection rates by method of chromosome analysis

| Type of Testing | N abnormal | N tested | % |
|---|-------------------|-----------------|----------|
| Total, any testing | 13 | 48 | 27.1% |
| Classical cytogenetic analysis AND Array CGH | 10 | 39 | 25.6% |
| <i>Detected by standard chromosome analysis</i> | 2 | 39 | 5.1% |
| <i>Detected by array CGH only</i> | 8 | 39 | 20.5% |
| Standard Chromosome Analysis | 4 | 47 | 8.5% |
| FISH (22q11.2) | 1 | 10 | 10% |
| Array CGH | 9 | 40 | 22.5% |
| <i>Abnormal, Clinically Significant</i> | 3 | 40 | 7.5% |
| <i>Unclear Clinical Significance</i> | 6 | 40 | 15% |

Of the fifty subjects enrolled in our study, 48 subjects (96%) received at least one type of chromosomal analysis. Of these subjects, 27% had some type of chromosome aberration. Of all subjects tested by classical cytogenetic analysis, 8.5% were found to have a chromosome abnormality, including a balanced translocation, a large deletion, Trisomy 21, and mosaic Trisomy 21. Of all subjects tested by array CGH, 22.5% (n= 9) had a CNV detected. Only one 22q11.2 microdeletion associated with DiGeorge syndrome was detected of the 10 subjects with FISH testing for 22q11.2, but it is important to note that any microdeletions in this region would have been detectable by array CGH as well. Therefore, out of the total number of individuals with FISH or array CGH (n=44), the detection rate for microdeletions of 22q.11.2 would be approximately 2%.

Due to the recent implementation of ordering both classical cytogenetic analysis and array CGH as standard of care for neonates with CHD, 39 subjects (78% of all subjects enrolled) received both classical cytogenetic analysis and array CGH. Of the subjects who received both tests, two subjects (5.12%) had a chromosome abnormality detected by classical cytogenetic analysis alone. Array CGH detected one of these chromosome abnormalities (a large deletion) but not the other (a balanced translocation, which may not be pathogenic). There were a total of 8 CNVs detected by array CGH which were not detected by classical cytogenetic analysis. Therefore, the addition of array CGH increased the detection of chromosome aberrations by 20.5%, to yield a combined detection rate of 25.6%. Of the CNVs detected by array CGH, approximately 33% (n=3) had clear clinical significance, while 67% (n=6) had unclear clinical significance.

McNemar's test was utilized to assess whether this increase in detection rate by array CGH is statistically significant. The null hypothesis was set as "the detection rates of array CGH and classical cytogenetic analysis are the same in our study population." The alternative hypothesis was set as "the detection rate of array CGH is significantly greater than the detection rate of classical cytogenetic analysis in our study population." This test analyzes paired data, and therefore only captures the 39 subjects who received testing by both array CGH and classical cytogenetic analysis. There were a total of 9 discordant pairs: 8 subjects had a CNV detected by array CGH but a normal karyotype, and 1 subject had normal array CGH results but an abnormal karyotype (balanced translocation). The two-sided p-value obtained using McNemar's test was 0.039. Therefore, we can reject the null hypothesis at a significance level of $\alpha=0.05$.

3.1.3 Phenotypic Features

Specific criteria used to categorize dysmorphic features and extracardiac abnormalities are discussed above in Section 1.4. Among our study population, there were a total of 21 subjects with abnormal findings on head or renal imaging studies and a total of 15 subjects with other extracardiac abnormalities such as congenital anomalies or other diagnoses. The combined frequency of any extracardiac abnormalities (including abnormal imaging studies and/or other extracardiac abnormalities) in our study population was 54% (n=27). The frequency of dysmorphic features among our study population was 60% (n=30). Of note, these findings were largely based on the assessment of Genetics providers, as 80% of our entire study population received some level of Genetics assessment. In contrast, when only including assessments by Cardiology providers, only 14% (n=7) of all subjects enrolled were described as having dysmorphic features, while the remaining 86% of subjects were described as having “no obvious deformities” or as “non-dysmorphic” in appearance.

Subjects with either dysmorphic features or extracardiac abnormalities were categorized as having apparently syndromic CHD, while subjects with neither dysmorphic features nor extracardiac abnormalities were categorized as having apparently isolated CHD. The term “apparently” is used to indicate that in cases with additional features, there may not truly be a syndromic cause, and in cases with no additional features, other clinical features may become apparent later in life and the CHD may not be truly isolated. According to these criteria, the frequency of apparently isolated CHD in our study population was 26% (n=13) and the frequency of apparently syndromic CHD in our study population was 74% (n=37). Figure 5

depicts the prevalence of syndromic versus isolated CHD in our study population, subdivided by the presence of extracardiac abnormalities or dysmorphic features.

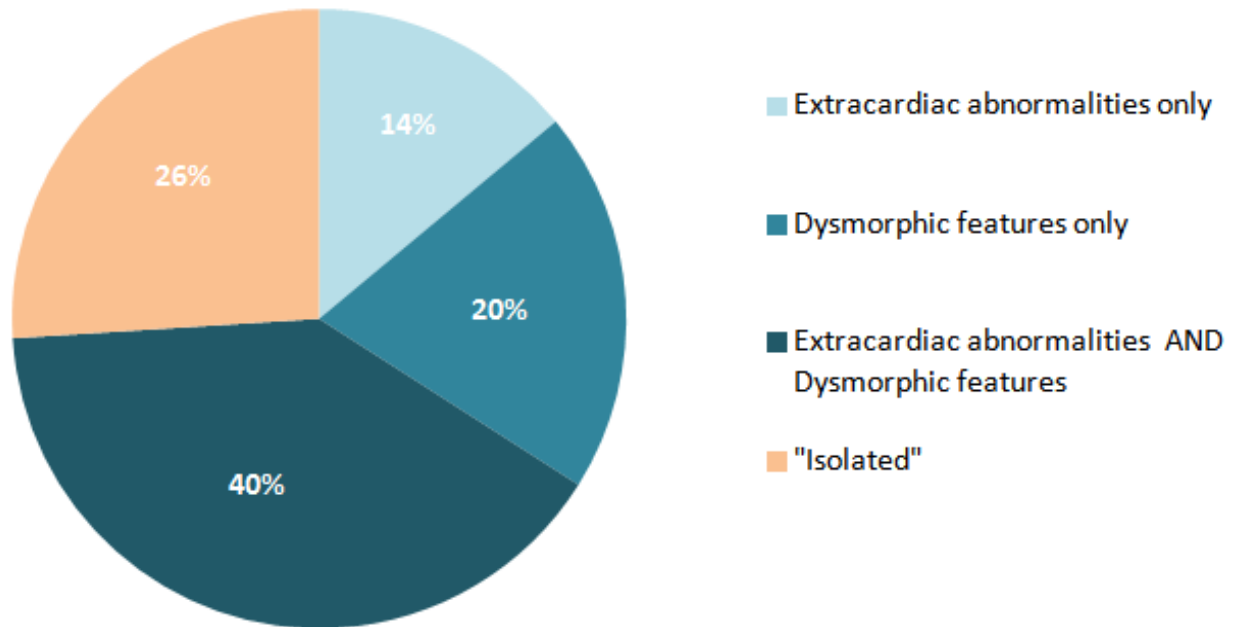


Figure 5. Prevalence of apparently syndromic versus apparently isolated CHD

The prevalence of apparently syndromic versus apparently isolated cases of CHD, subdivided by the prevalence of chromosome aberrations within each group, is depicted below in Figure 6. This includes chromosome aberrations detected by both classical cytogenetic analysis and array CGH. The frequency of chromosome aberrations among subjects with apparently isolated CHD was approximately 8% (n=1). The frequency of chromosome abnormalities among subjects with apparently syndromic CHD was approximately 32% (n=12).

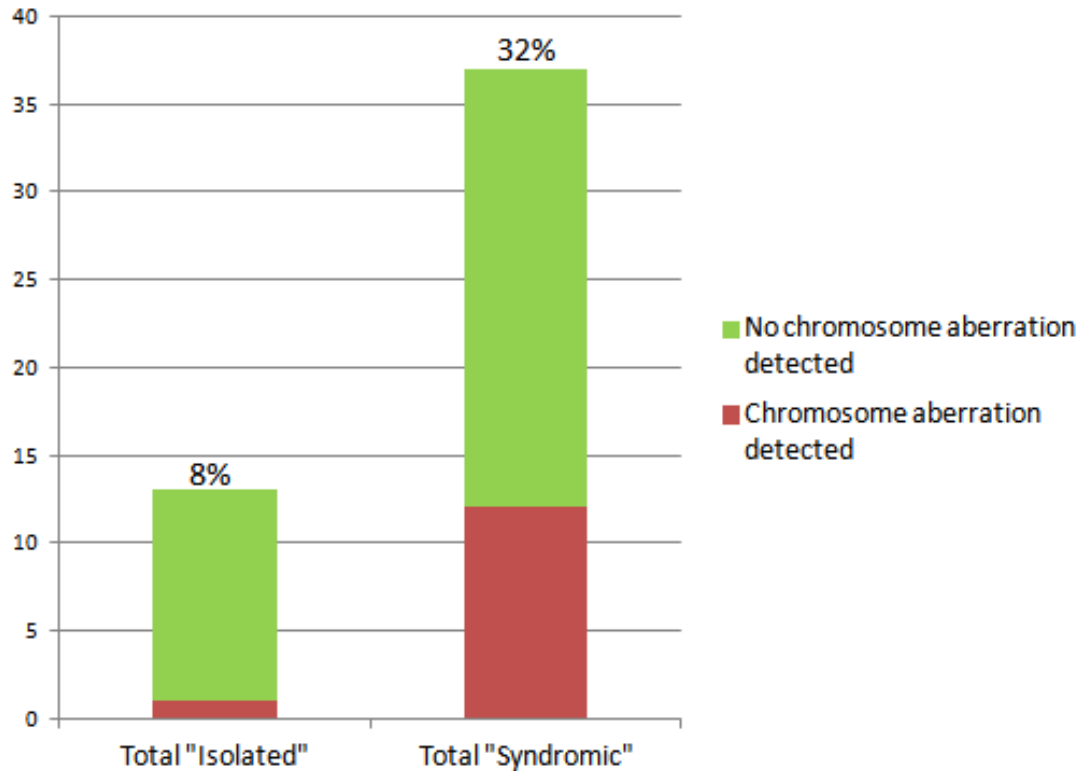


Figure 6. Frequency of chromosome aberrations among apparently syndromic and apparently isolated cases of CHD

Figure 7 depicts the subdivision of subjects with apparently syndromic CHD, based on the type of phenotypic features exhibited. Of subjects with apparently syndromic CHD, 19% (n=7) had only extracardiac abnormalities but no dysmorphic features noted; one (14%) of these subjects had a chromosome aberration. Subjects with only dysmorphic features but no other congenital anomalies, diagnoses, or abnormalities on imaging studies made up 27% (n=10) of subjects with apparently syndromic CHD; among these, 3 (30%) had a chromosome aberration. Subjects with both extracardiac abnormalities and dysmorphic features were the largest group, comprising 54% (n=20) of all subjects with apparently syndromic CHD; 8 (40%) of these subjects had a chromosome aberration.

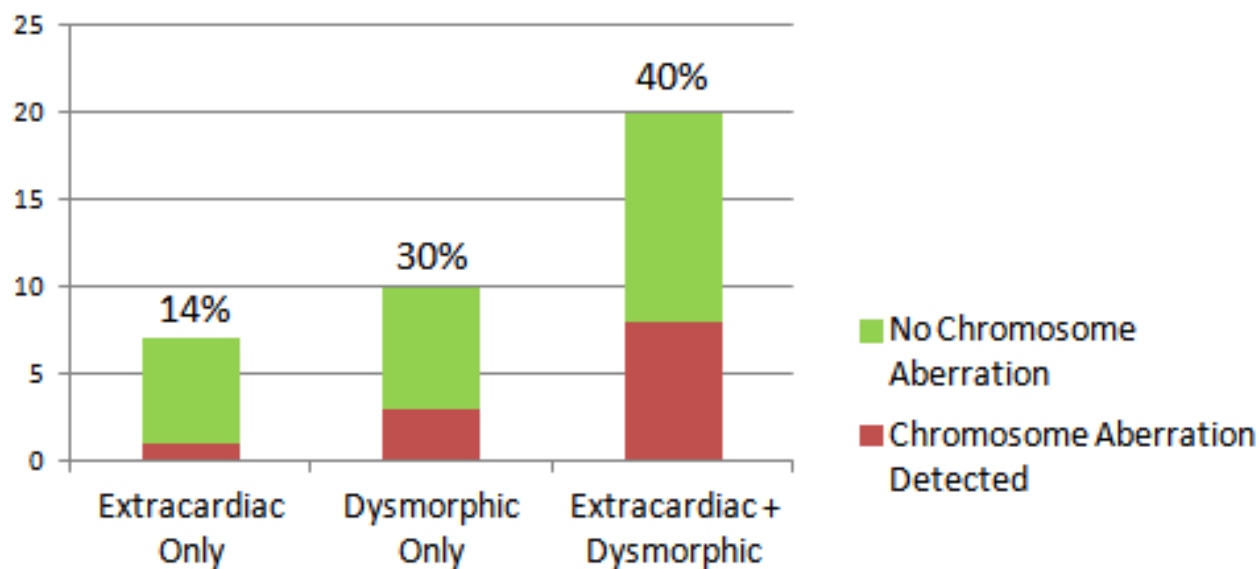


Figure 7. Frequency of chromosome aberrations in neonates with apparently syndromic CHD, based on phenotypic features

The frequencies of various types of CHD in our study population are presented below in Figure 8, as categorized by the Level III categories established by Botto et al. (18). This figure also depicts the prevalence of apparently syndromic versus apparently isolated heart defects within each group. Any associations or complex defects which had components in multiple categories are included under in “Other, Combined.”

Conotruncal defects comprised the largest group of heart defects represented in our study, accounting for 40% of all subjects enrolled. This prevalence would be even higher if it included the conotruncal defects which were a component of a complex defect. Left ventricular outflow tract obstruction defects (LVOTO) are the next largest group, accounting for 16% of all subjects. Right ventricular outflow tract obstruction defects (RVOTO) accounted for 8% of the study population. Six percent of the population had a septal defect, 10% had a septal and LVOTO

defect, 4% had a septal and RVOTO defect, and 14% had another combination of defects. These are not reported individually, as each combination of defects was unique.

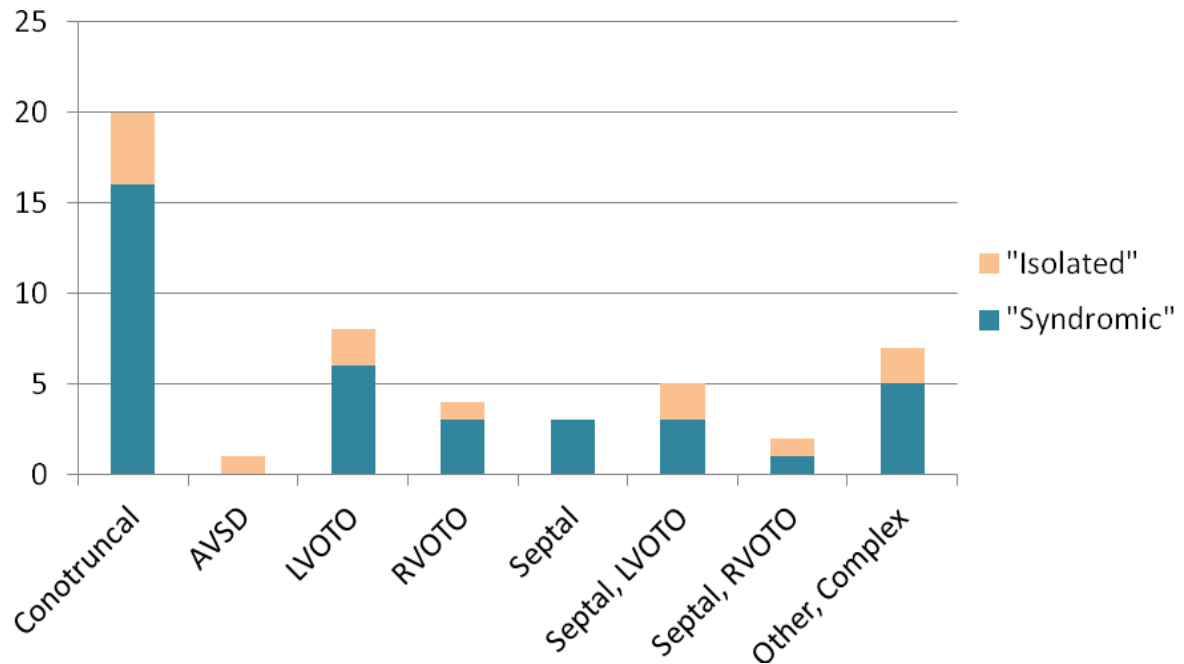


Figure 8. Frequency of each type of CHD subdivided by "syndromic" and "isolated" cases

Figure 9 depicts the prevalence of each type of CHD among the thirteen subjects with a chromosome aberration detected by array CGH and classical cytogenetic analysis. Again, conotruncal defects had the highest prevalence (38.5%) among these subjects.

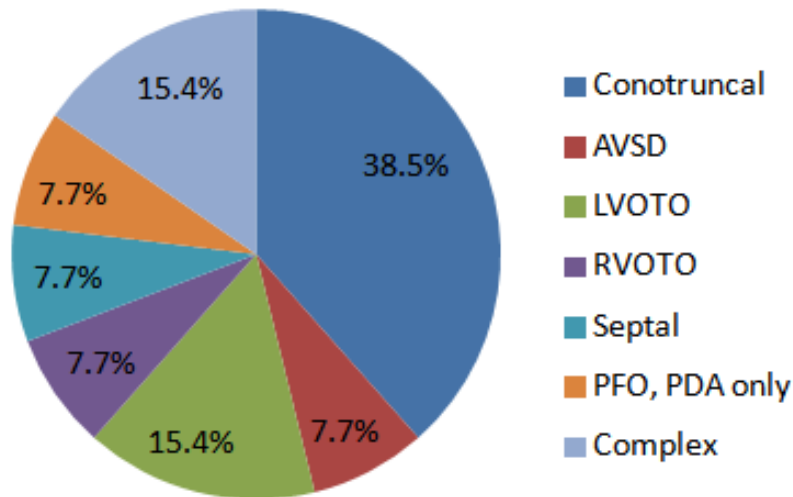


Figure 9. Frequency of each type of CHD among 13 subjects with a chromosome aberration

Due to the presence of syndromic features or abnormal genetic test results, 19 subjects in our study population received a full Medical Genetics consult. An additional 21 subjects received a baseline dysmorphology exam by a Geneticist per standard of care. Therefore, a combined total of 80% of subjects enrolled in the study received some level of Genetics assessment. Of the subjects who received a genetics evaluation, 9 had additional genetic testing for specific syndromes. Four had findings of unclear clinical significance, which are briefly discussed in Section 1.6. The remaining five subjects either had normal test results or tests which were pending at the time of this report. Of note, two subjects with findings of unclear clinical significance by array CGH did not receive a formal Genetics consult.

3.1.4 Chromosome Aberrations

All chromosome aberrations detected in this study, including large abnormalities detected by classical cytogenetic analysis and CNVs detected by array CGH, are recorded in Table 3.

This table includes basic information used in the assessment of pathogenicity of each CNV, such as size and chromosomal location, inheritance, number of OMIM genes in the region of genomic imbalance, and availability of case reports of similar CNVs. A more detailed assessment of causality for each CNV detected by array CGH is included in Appendix A.

Phenotypic features of all patients with a chromosome aberration detected by classical cytogenetic analysis or array CGH are presented in Table 4, including categorization of each subject's CHD, type of phenotypic features present, and any abnormalities detected on imaging studies. These features are discussed in greater detail in Appendix A.

Table 4. Description of all chromosome aberrations detected

| Patient ID | Region | Gain/Loss | Size (Mb) | Start | End | Inheritance | # OMIM Genes | Previous Case Reports | Clinical Significance per Lab |
|--------------------------------|-------------------------|-----------------|---|-------------|-------------|-------------|--------------|-----------------------|-------------------------------|
| Array CGH | | | | | | | | | |
| 110 | 3p23p22.2 | Loss | 7.7 | 30,940,540 | 38,605,621 | -- | 35 | Yes | Abnormal |
| 118 | 16q21 | Gain | 0.683 | 57,098,728 | 57,781,710 | M | 2 | No | VUS ^c |
| 119 | 3p26.3 | Loss | 0.295 | 1,124,286 | 1,419,226 | -- | 1 | No | VUS |
| 121 | 1q21.1 | Gain | 1.19 | 144,998,070 | 146,193,043 | -- | 9 | Yes | Abnormal |
| 127 | 2q21.3 | Loss | 0.358 | 135,489,180 | 135,847,346 | -- | 2 | No | VUS |
| 128 | 16p11.2 | Loss | 0.535 | 29,564,890 | 30,100,123 | -- | 18 | Yes | Abnormal |
| 132 | 22q12.3 | Loss | 0.346 | 32,191,531 | 32,537,463 | P | 1 | No | VUS |
| 140 | 3p14.1 | Loss | 1.36 | 67,719,379 | 69,080,808 | -- | 2 | No | VUS |
| 141 | 20p11.21 | Gain | 0.398 | 25,016,046 | 25,414,542 | -- | 5 | No | VUS |
| Classical Cytogenetic Analysis | | | | | | | | | |
| 102 | 46,XX,t(6;10)(p21.2;15) | BT ^b | --- | | | P | NA | -- | ND |
| 110 | 46,XY,del(3)(p22.2p23) | Loss | Also detected by array CGH, listed above | | | -- | 35 | Yes | Abnormal |
| 126 | Mosaic Trisomy 21 | Gain | Prenatal diagnosis, level of mosaicism undetermined | | | -- | NA | Yes | Abnormal |
| 143 | Trisomy 21 | Gain | Prenatal diagnosis | | | -- | NA | Yes | Abnormal |
| FISH for 22q11.2 | | | | | | | | | |
| 148 | Microdeletion 22q11.2 | Loss | Prenatal diagnosis | | | -- | -- | Yes | Abnormal |

^a M- maternal; P- paternal, -- not determined

^b Balanced Translocation

^c VUS- Variant of Uncertain Significance

Table 5. Phenotypic features of patients with chromosome aberrations

| Patient ID | Sex | Types of CHD ^a | | | Phenotypic Features Extracardiac Features or Dysmorphic Features | Imaging Studies | |
|------------|-----|--|---|-------------|---|-----------------|-----------------|
| | | Level I (Detailed) | Level III (Broad) | Complexity | | Head U/S or MRI | Renal U/S |
| 102 | F | TOF | Conotruncal | Simple | Extracardiac | Normal | Normal |
| 110 | M | Double Outlet Right Ventricle, TOF type Pulmonary Valve Stenosis | Conotruncal | Simple | Dysmorphic | Normal | ND ^a |
| 118 | M | D-TGA with noninlet VSD and RVOTO (AS and COA) Double Outlet Right Ventricle, TGA type | Conotruncal | Association | Both | Abnormal | Abnormal |
| 119 | M | ASDs, multiple | Septal | Simple | Both | Normal | Abnormal |
| 121 | F | HLHS with intact ventricular septum | LVOTO | Simple | Dysmorphic | Normal | Normal |
| 126 | M | AVSD with RVOTO HLHS with intact ventricular septum DORV, TGA type | AVSD LVOTO Conotoruncal | Complex | Dysmorphic | Normal | Normal |
| 127 | F | Patent Ductus Arteriosus, Patent Foramen Ovale | -- | Simple | Both | Normal | Abnormal |
| 128 | F | TOF | Conotruncal | Simple | Both | Normal | Abnormal |
| 132 | F | Single Ventricle, Double Inlet Left Ventricle type, nos Pulmonary Valve Stenosis VSD | Single Ventricle/Complex RVOTO Septal | Complex | Both | Normal | Normal |
| 140 | F | Atrioventricular Septal Defect with RVOTO | AVSD | Simple | None | Normal | Normal |
| 141 | M | HLHS | LVOTO | Simple | Both | Normal | Abnormal |
| 143 | M | VSD Pulmonary valve stenosis | RVOTO | Association | Extracardiac | Abnormal | ND |
| 148 | F | Interrupted aortic arch, type B VSD, conoventricular type | Conotruncal | Association | Both | Abnormal | Abnormal |

^a Type of CHD is presented based on Level I (detailed) and Level II (broad) category [Botto].

^b Not done

4.0 DISCUSSION

The question of whether array CGH should be included as a first-tier test for neonates with CHD is complex. The spectrum of data collected for this study, and from previous studies done to date, is broad and there are many factors to consider in the interpretation of results. This discussion provides a comprehensive review of the significance of our results, regarding the contribution of chromosome aberrations as one cause of CHD and the efficacy of array CGH as a first-tier test among neonates with CHD. The conclusions drawn from our results which address the primary aims of the study are presented below. The strengths and limitations of our study, ethical considerations, and future directions are discussed in Sections 2.1.4 and 2.1.5.

4.1.1 Aim 1: Comparison of Detection Rates

One aim of our study was to determine if the prevalence of chromosome abnormalities detected by classical cytogenetic analysis matched the 14% detection rate which was previously reported at our institution (43). Of all subjects tested by classical cytogenetic analysis, the detection rate by this method of chromosome analysis alone was only 8.5%. Although this is a lower prevalence of large chromosome abnormalities than previously reported at our institution, it is a reasonable prevalence in comparison with the literature prevalence of 8 to 13% of large chromosome abnormalities among individuals with CHD (2, 3). It is also important to note that

the total detection rate of chromosome aberrations among our study population was 27.1%, as detected by classical cytogenetic analysis, FISH, and array CGH combined. This includes all findings, regardless of clinical significance, but still provides strong evidence that chromosomal causes are very important to the overall etiology of CHD and warrant further research.

The primary aim of our study was to determine whether array CGH significantly increases the detection rate of chromosome abnormalities in comparison to classical cytogenetic analysis. We hypothesized that the addition of array CGH would increase detection by 6% over the previously reported 14% detection rate by classical cytogenetic analysis alone among neonates in the CICU at our institution (43), to yield a total detection rate of at least 20%. Our actual detection rate was even greater than hypothesized: among all subjects receiving both tests, the actual combined detection rate was 25.6%. Array CGH increased detection by 20.5%, as only 5.1% of the subjects receiving both tests had a chromosome abnormality detected by classical cytogenetic analysis. In addition, the total detection rate of CNVs among all subjects in our study tested by array CGH (22.5%) was very similar to that which was previously reported by Lu et al. among neonates with CHD (21.8%) (15). Using McNemar's test of significance, we obtained a p-value of 0.039, which reflects a low probability of observing our data under the null hypothesis of equal detection rates. We can reject the null hypothesis, and conclude that within this population of neonates with CHD, the detection rate of array CGH is significantly greater than that of classical cytogenetic analysis alone.

There are several limitations of our population which may have affected the statistical analysis of our data. First, our sample size was relatively small: a total of 50 subjects were enrolled of the 59 patients who were eligible for participation. In two cases, the parents declined participation in the study. In three cases, the parents were contacted but were discharged prior to

signing the consent; in some cases parents were contacted via telephone but consent forms were not returned by mail. Three patients passed away during their admission to the CICU and we elected not to contact these parents regarding participation. One patient could not be enrolled due to a language barrier and the lack of a consent form in the native language of the parents.

Second, there was a significant lack of consistency in ordering array CGH. The standard of care to order both classical cytogenetic analysis and array CGH upon admission is very recent and was being implemented only as the study commenced. Therefore, only 78% of all subjects enrolled received both tests. The remainder received only one or neither test; some received FISH for 22q11.2 instead of array CGH. Because the test results can be categorized as non-independent, paired binomial data which is best represented in a contingency table, McNemar's was the most pertinent to our analysis. However, due to the paired nature of this test, our effective sample size was limited to 39 subjects. Results from subjects who did not receive both tests were non-paired and could not be captured.

In addition, three subjects had chromosome abnormalities detected prenatally by classical cytogenetic analysis or FISH analysis and did not receive testing by array CGH and therefore could not be captured in our statistical analysis. These subjects had Trisomy 21, mosaic Trisomy 21, and microdeletion 22q11.2. Had the subjects received chromosome analysis by array CGH, it is very likely that each abnormality would have been detected by array, with the possible exception of mosaic Trisomy 21. By assuming all of these subjects had abnormal array CGH results, the number of discordant pairs detected by array CGH only would have increased to 9 due to the 22q11.2 microdeletion, which would have lowered the p-value to 0.013. By assuming each abnormality except mosaic Trisomy 21 would have been detected, the number of discordant pairs detected by classical cytogenetic analysis would have also increased to 2, and increased the

p-value to 0.041. However, in either scenario, our p-value would have still been statistically significant to reject the null hypothesis.

We report a significant increase in detection rate by utilizing array CGH among neonates with CHD. It is important to recognize that this detection rate is within a unique study population, which does not include children or infants who were diagnosed after 4 weeks of life. It also does not include miscarriages, stillborns, or infants admitted to the CICU after 4 weeks of age, which may have milder cases of CHD. Additionally, neonates with an aneuploidy, such as Trisomy 13 or 18, are likely to receive palliative care instead of surgical treatments and therefore are not likely to be admitted to the CICU.

Another important consideration is that our detection rate incorporates all CNVs detected, both with known clinical significance and unclear clinical significance. All results are included because of the uncertainty which is intrinsic to interpreting the clinical significance of CNVs. CNVs with unclear clinical significance may in fact be causal for the patient's CHD, but the available data may not be sufficient to draw this conclusion at the present time. Thus, it is important to identify and reassess the potential causality of each CNV over time as more genes implicated in CHD are identified. In contrast, CNVs with known clinical significance may not be causal for the patient's CHD, but may still be clinically relevant and potentially causal for additional syndromic features. This is exemplified by patients 121 and 128 (see appendix A). Because we have reported all CNVs, this detection rate is likely inflated above the actual detection of pathogenic results. However, it is important to consider the clinical impact of the total detection rate, because all cases in which a CNV is detected will warrant the assessment of a geneticist to evaluate the clinical significance and consider additional genetic testing.

4.1.2 Aim 2: Clinical Significance of Array CGH Results

Case reports for all chromosome aberrations detected by array CGH are presented in Appendix A. There are several trends among these case reports which illustrate both the advantages and the clinical challenges of utilizing array CGH in this population. There were several subjects with a CNV detected only by array CGH, which has been previously associated with disease and therefore provides clinically relevant information for both the physicians and the patient's family. However, approximately two-thirds of the CNVs detected in our population by array CGH had unclear clinical significance. Interpreting the clinical significance of results may pose a significant challenge when there is a limitation of data which can aid in interpretation. In addition, even among CNVs which are clearly pathogenic, it may still be difficult to determine whether the CNV is causal for the heart defect or only the additional extracardiac abnormalities.

One factor which limited the interpretation of CNVs in our study was the lack of parental testing, which can be attributed to multiple causes. In some cases, the patient did not receive a Genetics consult and may not have been offered parental studies. Other subjects were offered testing, but have not yet elected to pursue testing at the time of data collection; if the data is re-analyzed in the future, these parental studies may be completed and may aid in interpretation. Another factor which currently limits the interpretation of array CGH results is that databases of CNVs detected in disease and healthy populations are still relatively new. When the CNV has not been previously reported in any databases, it can be very difficult to interpret significance. As array CGH continues to be utilized among patients with various indications, the available data which can be used for interpretation will certainly increase as well.

To address the challenges of interpreting array CGH results, the involvement of Medical Genetics is crucial. A physical exam by a geneticist specializing in dysmorphology may be helpful in determining the syndromic or isolated nature of the CHD. If it appears to be syndromic in nature, it is important to consider whether the CNV is likely causal for the syndromic features or whether the features are suggestive of a specific monogenic disorder, in which case additional genetic testing can be ordered. If case reports of similar CNVs exist in the literature, the phenotypic features of the patient can be compared with previous reports. Finally, the involvement of Medical Genetics in cases with a VUS will ensure that parental studies are offered, recurrence risk is discussed, and appropriate follow-up is provided to re-evaluate phenotypic features and the potential causality of the CNV in the future.

Despite these challenges, there are many advantages of using array CGH as a first-tier test. First, one third ($n=3$) of all CNVs detected by array CGH were clearly abnormal, for a detection rate of 7.5% for clinically significant CNVs among all subjects tested by array CGH. Not all of these CNVs were necessarily causal for the patient's CHD, but each had definite clinical significance. Patient 110 had a large deletion of 3p23p22.2 which was also detected by classical cytogenetic analysis; detecting this deletion led to the diagnosis of several monogenic syndromes. It was likely causal for the patient's CHD, as several genes in this region have been associated with heart defects. Patient 121 was diagnosed with 1q21.1 duplication syndrome; this syndrome is associated primarily with DD/ID and dysmorphic features, but there have been case reports of heart defects (aortic valve abnormalities) associated with 1q21.1 duplication syndrome (58). Patient 128 was diagnosed with 16p11.2 microdeletion syndrome. This syndrome is also associated with neurologic manifestations, primarily autism spectrum disorder; there has been at least one case report of a heart defect associated with this syndrome (59). Each of these cases is

discussed in greater detail in Appendix A. In each of the above cases, the use of array CGH provided valuable information for the continued monitoring of these patients.

In cases in which a pathogenic CNV is detected by array CGH, the early diagnosis can provide valuable information regarding recurrence risk and sometimes medical management information for both the patient and their family members. If the CNV is determined to be parental in origin, the recurrence risk would be 50% for each subsequent pregnancy; this is significantly different from the standard 2 to 5% recurrence risk provided based on an assumption of multifactorial etiology. One example in which critical management information was provided for both the patient and their family was Patient 110. This patient had a deletion including genes associated with cardiac conduction defects, as well as *MLH1*, associated with Lynch syndrome. Parental studies could not be completed immediately following the patient's diagnosis, but because of the clinical severity of the disorders associated with this deletion, a baseline cardiology evaluation and colonoscopy was recommended for all at-risk family members until further testing could be completed.

Even the detection of a VUS can provide clinically useful information. In many of the cases discussed in Appendix A, there were no previous reports of similar CNVs, gene dosage effects were unclear, and parental studies were not completed. Therefore, it is still possible that the CNV is pathogenic but the mechanism is unknown; the continued monitoring of the patient for any additional syndromic features is still important. If additional phenotypic features become apparent later in life, the patient can be re-evaluated to determine if the CNV is likely causal or if additional genetic testing is warranted. In many cases within our study, patients with a VUS did present with additional phenotypic features which could indicate a syndromic etiology.

In addition, there are some cases in which a deletion with unclear clinical significance encompasses a gene associated with an autosomal recessive disorder. In most cases, this patient is simply an asymptomatic carrier for this disorder. However, in some rare cases, the patient may carry a mutation in the other allele and would be affected. For this reason, once such a deletion has been identified, this patient can be monitored in the case that phenotypic features indicating a mutation in the other allele would become apparent. In these rare cases, early detection of the CNV may greatly minimize the time needed to reach a diagnosis. Knowing carrier status for the disorder may also be useful for the patient in the future at the time of child-bearing. If testing is clinically available, their partner would have the option of genetic testing for mutations in that gene in order to determine their risk of having a child with the disorder.

4.1.3 Aim 3: Phenotypic Features

The third aim of our study was to evaluate the relationship between chromosome abnormalities and phenotypic features among our study population. Phenotypic features, including the type of heart defect and the presence of dysmorphic features and other extracardiac abnormalities, have been frequently used in the past as criteria to determine which patients should receive genetic testing. Classical cytogenetic analysis and array CGH are now utilized as first-tier tests for any neonates with CHD at our institution regardless of additional phenotypic features. However, the presence of syndromic features is still important in determining which patients should receive a Medical Genetics consult and whether additional investigation into monogenic syndromes is warranted.

The majority of subjects enrolled in our study did exhibit additional findings in the neonatal period, including either dysmorphic features or other extracardiac abnormalities, to yield a very high prevalence (74%) of apparently syndromic CHD. In comparison, it has been reported in previous literature that 25 to 40% of patients with CHD have additional birth anomalies (7, 11). Several additional factors are likely to have contributed to the high prevalence of apparently syndromic CHD in our population. First, by virtue of admission under four weeks of age, the neonates included in our study have a more severe clinical presentation, which may increase the frequency of syndromic CHD compared to a population of individuals diagnosed at a later age with less severe CHD. Second, our criteria for assessing whether any syndromic features were present were quite broad in order to maintain consistency and avoid subjectivity. Not all subjects enrolled received a full evaluation by a geneticist, but any subject with any dysmorphic features and/or extracardiac abnormalities were considered to have syndromic CHD. In addition to congenital anomalies, we included any abnormalities detected on imaging studies, additional diagnoses, or features noted to be “abnormal” on a dysmorphology exam by a geneticist. These broader criteria ensure the inclusion of all individuals with features suggestive of a syndromic etiology. Sometimes these features are unclear in the neonatal period, and therefore the disadvantage to these criteria is that patients may be included who will later be considered to have isolated CHD.

Based on our data to this point, array CGH had a much greater detection rate among patients with apparently syndromic CHD. Of the 13 subjects with a chromosome aberration, 12 were categorized as having apparently syndromic CHD based on our criteria. Three of the subjects with apparently syndromic CHD and a chromosome aberration detected had dysmorphic features but no other extracardiac abnormalities, while only one had extracardiac abnormalities

but no apparent dysmorphic features. Although our sample of patients with chromosome aberrations was quite small, these findings suggest that dysmorphic features, as identified by a geneticist, may be equally as useful as extracardiac abnormalities in predicting whether a chromosome abnormality will be detected.

Regarding the prevalence of dysmorphic features among our population, 60% of the entire study population had at least one abnormal feature on an exam by a geneticist, and 20% had only dysmorphic features but no extracardiac abnormalities. This prevalence may have been even higher had all subjects received an exam for dysmorphology. Some of these patients may not have an overall dysmorphic appearance, but did have features noted to be abnormal which warrant reassessment in the future. However, it is important to note that, although 60% of our study population had dysmorphic features when assessed by a geneticist, only 14% were noted to have dysmorphic features in the clinical notes by Cardiology providers. This discrepancy may be attributed to the fact that features can be very subtle, especially in the neonatal period, and may only be recognized by a geneticist who has expertise in dysmorphology.

The increased detection of CNVs among subjects with apparently syndromic CHD could lead to the conclusion that only neonates with additional features should receive array CGH as a first-tier test, similar to recommendations made in previous studies for older individuals with CHD. However, this conclusion could be problematic for several reasons. First, the prevalence of syndromic CHD was 74% among our population; the increased detection rate of CNVs among this group may simply reflect this distribution. This trend may not persist if the criteria for the categorization of apparently syndromic CHD were more stringent. In addition, the detection of dysmorphic features in our population was much greater when based on the assessment of a geneticist. For example, Patients 110 and 121 both had a pathogenic CNV and had dysmorphic

features which were only identified by a geneticist. Basing testing on the presence of syndromic features, especially among patients who are not evaluated by a geneticist, may result in missing patients with truly syndromic CHD caused by a chromosome abnormality.

As exemplified by our results, both dysmorphic features and other extracardiac abnormalities are important in assessing syndromic versus isolated CHD. To identify occult extracardiac features, it has been previously recommended that all patients with CHD receive a routine head ultrasound and renal ultrasound (43). It would be ideal for every neonate with CHD to have an evaluation by a geneticist to identify dysmorphic features and assess whether the CHD appears isolated or syndromic in nature. However, it may not be feasible for every infant with CHD to receive a Medical Genetics consult. One reasonable approach may be to have any neonate with CHD and congenital anomalies, abnormal imaging studies, or suspected dysmorphism receive a physical exam by a geneticist to determine if any testing in addition to classical cytogenetic analysis and array CGH is warranted.

4.1.4 Additional Challenges and Considerations

Through the course of our study, several systemic challenges were discovered which may have direct impact on the efficacy of array CGH in this population. First, not all subjects were tested by both array CGH and classical cytogenetic analysis as this is a newly implemented standard of care. In addition, although a large portion of our study population (38%) did have a formal inpatient genetics consult due to the presence of syndromic features or abnormal genetic test results, there were two patients with a VUS for which a Medical Genetics consult was not ordered. Without the involvement of Medical Genetics, parental studies may not be obtained,

which limits interpretation of results, and the family may not be provided with appropriate counseling and follow-up. These findings suggest that if array CGH is to be ordered by cardiology providers as a first-tier test, it is important to establish an algorithm which would ensure that both tests are ordered and that families are provided with proper follow-up for abnormal results.

Another challenge which became apparent through our study was the lack of consistent entry of genetic results in electronic medical records. This may have been one factor which contributed to the instances in which patients with a VUS did not have a Medical Genetics consult, as the results of genetic testing may not have been brought to the attention of the attending physician. Any genetic test reports are sent to the ordering physician, but are not frequently listed in the part of the electronic medical record system which is utilized by most providers for the most up to date clinical records. When there is a transfer of care from the CICU providers to the NICU or Cardiology, the current physician may not have electronic access to the results. The laboratory is currently working on developing a solution to this problem, which will likely include the direct entry of all genetic test results from the laboratory into the electronic medical record system, to ensure ease of access by all healthcare providers.

One important consideration when utilizing array CGH among neonates with CHD is that this test cannot detect any single gene defects. Tests for specific monogenic disorders may be warranted in addition to array CGH when the CHD appears to be syndromic but it cannot be attributed to a chromosomal etiology. To this point, eight subjects in our study received additional genetic testing. Of these, three had normal test results and one had tests pending at the time of this report. Four had a VUS on single gene testing. Of these, two were detected on a Pancardio Panel. One of these patients had a VUS in three genes including *ANKRD1*, *CRYAB*,

and *TTN*; all of which are expressed in cardiac muscle and have been implicated in cardiomyopathy. The other patient had a VUS in the gene *DSG2*. This VUS was noted to be likely pathogenic, and mutations in this gene are associated with forms of arrhythmogenic right ventricular dysplasia and dilated cardiomyopathy (60). This subject was diagnosed with dilated cardiomyopathy and two ASDs. Another subject had clinical indications including dysmorphic features, lax skin, tortuous carotid arteries, and umbilical hernia along with a VSD and COA. This subject had a sequence variant detected in *TGFBR1*, one gene associated with Loeys-Dietz syndrome; this variant resulted in the missense substitution of an aspartic acid with a tyrosine. Another subject had a sequence variant in the gene *G6PC* which is associated with glycogen storage disease type IA; this VUS was considered unlikely to be pathogenic. This is an autosomal recessive syndrome, so if the VUS were pathogenic, this subject would only be a carrier for the disorder (60).

There has recently been debate regarding whether array CGH should replace classical cytogenetic analysis as the only first-tier test among patients with various clinical indications. The benefits of utilizing array CGH only is that the cost would be substantially lower and the majority of causal abnormalities would be detected; in our study at least 11 of the 13 total chromosome aberrations would have been detected by array CGH alone. However, two diagnoses may not have been made, including mosaic Trisomy 21, which was likely causal, and a balanced translocation, which may not have been causal. The detection of mosaic Trisomy 21 is dependent on the level of mosaicism; this patient (Patient 126) did not have array CGH so it is not clear whether it would have been detected. The detection of balanced translocations does provide very important reproductive information for parents due to the risk for unbalanced translocations. In other cases, balanced translocations may be causal for CHD due to the

disruption of important genes. Our sample size is too limited to draw conclusions on this matter, and further studies with a larger sample size are warranted to determine the optimal testing strategy. One possible solution would be to utilize classical cytogenetic analysis as a second-tier test in cases with normal array CGH results but features suggestive of a chromosomal syndrome (i.e. parents with multiple miscarriages); this may maximize cost effectiveness with a minimal decrease in the efficiency of diagnosis. At this point, it seems that the most conservative strategy to ensure the detection of causal chromosome abnormalities among neonates with CHD is the simultaneous use of classical cytogenetic analysis and array CGH.

Various ethical and psychosocial considerations were exemplified by this study and are important to recognize when ordering array CGH on a clinical basis. First, array CGH has the potential to detect adult-onset conditions in a child with an unrelated clinical indication. One example in our study was that one subject (Patient 110) received the diagnosis of Lynch syndrome, also known as Hereditary Non-Polyposis Colorectal Cancer Syndrome, due to a deletion encompassing the gene *MLH1*. This is an adult-onset cancer predisposition syndrome. In families affected by Lynch syndrome, testing is not generally recommended until age 18 when the individual provides informed consent, as there are no associated childhood cancers. De novo inheritance was suspected in this case, but it has not yet been confirmed by parental studies. Due to the absence of a history of Lynch syndrome cancers in this family, it is unlikely that the diagnosis would have ever been suspected. The advantage of making this diagnosis via array CGH is that an increased screening regimen will be recommended for this patient in the future, which may lead to the prevention of a malignancy. Increased screening was also recommended for at-risk family members until additional testing provides confirmation that the deletion was de novo. The disadvantage of making this diagnosis is that it may cause a psychological burden on

the patient and his parents when no steps to reduce cancer risk can be taken at the present. This information could be undesired or overly burdensome for parents who bear the additional burden of coping with the diagnosis of CHD.

An important psychosocial aspect of the clinical use of array CGH is that making sense of uncertain results is challenging not only to the physicians but also to the patient's family. It may be very difficult for families to understand the meaning of a VUS. Even among subjects who carry a CNV associated with a contiguous gene syndrome, the variable expressivity and reduced penetrance which are characteristic of these syndromes can be difficult concepts for families to understand. In these cases, recurrence risk could be as high as 50%. However, in actuality it may be much lower due to reduced penetrance, but the exact degree of penetrance may not be well-defined. These families are already coping with a very stressful situation including the critical illness of their child and a strenuous course of hospitalizations and surgeries. In these cases, the genetic counselor plays a crucial role by explaining these challenging concepts with clarity and empathy and providing appropriate follow-up. Research on the psychosocial impact on parents from receiving results with uncertain significance within this population is warranted.

4.1.5 Future Directions

There are various limitations to our study which can be addressed in the future through continued enrollment, data collection, and analysis. One limitation was that this study was completed within the span of one year, which limited both the sample size and the availability of data from parental studies and follow-up genetics appointments. In several cases with apparently

syndromic CHD, additional genetic testing for monogenic disorders was not yet completed at the time of data collection. If this study is continued, the results of any additional genetic testing can be included to determine if any subjects, specifically those with a VUS detected by array CGH, have been given additional diagnoses which may explain their CHD.

The total sample size (n=50) and the small sample size of subjects who received both classical cytogenetic analysis and array CGH (n=39) are both relatively small. We plan to continue enrolling subjects in order to increase the total sample size. In addition, the percentage of subjects receiving both tests should increase over time as this standard of care becomes more established. We will reanalyze the data in the future to determine if the increase in detection by array CGH remains as high as the detection rate based on the data to this point. A larger sample size will facilitate a more statistically significant analysis of the prevalence of CNVs with clinical significance versus uncertain clinical significance. In addition, type of heart defect did not seem to have predictive value for isolated versus syndromic CHD, as the distribution of syndromic and isolated cases by type of CHD was fairly similar across all categories (Figure 8), but a larger sample size would facilitate additional investigation. A larger sample size would also allow us to determine whether the prevalence of CNVs varies by type of CHD.

Finally, we reported a prevalence of syndromic CHD which is higher than previously reported. We initially expected a prevalence of syndromic CHD which was lower than previously reported among all individuals of all ages with CHD, because neurologic features may not be apparent and dysmorphic features may be subtle during the neonatal period. Our results could indicate that many neonates with CHD have some additional, but sometimes subtle, syndromic features. In this case, fewer patients may have truly isolated CHD than suggested by previous studies. This may, in large part, be attributed to our study population of neonates which

are likely to have severe cases of CHD due to admission to a large tertiary care center. Our results could also be attributed to our broad criteria for categorizing apparently syndromic CHD. The criteria for the assessment of apparently syndromic CHD may be made more stringent in the continuation of this study to determine if the prevalence remains as high. By reassessing the detection rate of CNVs in a larger sample size with more stringent criteria for syndromic CHD, we will also be able to determine if the detection rate remains significantly greater among patients with apparently syndromic CHD. This may help to define characteristics of the population among which array CGH will have the greatest detection of pathogenic CNVs.

4.1.6 Conclusion

The results of our study support the conclusion that array CGH has significant clinical efficacy as a first-tier test among neonates with CHD. Our data to this point suggests that array CGH increases detection rate by approximately 20% in this population, compared to classical cytogenetic analysis alone. However, interpretation of the significance of results can be challenging, and the majority of CNVs detected in our study had unclear clinical significance. Further analysis is warranted in a larger sample size to clarify the detection rate of CNVs with clinical significance versus uncertain clinical significance. As array CGH continues to be utilized among neonates with CHD, the identification of additional genes associated with CHD and the expansion of databases of pathogenic CNVs will facilitate result interpretation. Array CGH does not always provide a clear answer regarding causality and recurrence risk for CHD, and additional testing for monogenic disorders may be required in cases which are suggestive of a particular syndrome. Therefore, the collaboration between Cardiology and Medical Genetics is

critical in cases with CNVs of uncertain clinical significance. Despite these challenges, array CGH does have the potential to detect pathogenic CNVs that are undetectable by standard methods of chromosome analysis. In these cases, the information provided by array CGH to patients, physicians, and families is invaluable.

APPENDIX A: CASE REPORTS

COPY NUMBER VARIANTS DETECTED BY ARRAY CGH

4.1.7 Case 110

Case 110 is a male with mixed ethnicity (African American, Italian, and Greek), born at 39 weeks of age. This patient was diagnosed with Double Outlet Right Ventricle, Tetralogy of Fallot type and severe PS. He was small for gestational age, with growth parameters at age 4 weeks of 7% for weight, 1% for length, and 2% for head circumference. No obvious deformities were noted by cardiology. On an exam by a geneticist, minimal dysmorphology was noted, including ears which were low-set and posteriorly rotated as well as excess skin on the neck related to a possible cystic hygroma. Phenotypic features noted to be normal variation included round facies and inverted nipples; the remainder of the physical exam was normal.

Classical cytogenetic analysis revealed a large deletion of approximately 7.7 Mb in size at chromosome 3p22.2 to p23; array CGH confirmed this deletion and defined the breakpoints (see Table 3). Factors considered in the assessment of clinical significance included:

- Previous Reports: Two deletion 3p syndromes have been reported: deletion 3p syndrome with breakpoints between 3p24 and 3p25 (which would not overlap this region) and

proximal deletion 3p syndrome with various breakpoints between 3p11 and 3p21.2 (a much larger deletion which overlaps this region). There is one case report of an interstitial deletion which overlaps with the region of interest, spanning from 3p22.2 to 3p24.2. Features noted in this case included global developmental delay, mild dysmorphic features, and short stature (61).

- Gene Content: Thirty-five OMIM genes are included in the deleted region, including seven genes with known disease associations: *MLH1*, *ACVR2B*, *GPD1L*, *SCN5A*, *GLB1*, *CRTAP*, and *MYD88* (56).
 - Heterozygous mutations in *ACVR2B* are associated with Visceral Heterotaxy-4, resulting in the abnormal placement of abdominal organs; this syndrome has been associated with various types of CHD (60).
 - Heterozygous mutations in *GPD1L* and *SCN5A* are associated with multiple syndromes involving the sodium channel of the heart. These disorders include Familial Atrial Fibrillation 10, Brugada Syndrome I and II, Dilated Cardiomyopathy IE, Heart block (nonprogressive and progressive type IA), Long QT syndrome 3, Familial ventricular fibrillation 1. Each of these disorders have reduced penetrance (60). To our knowledge, the effect of whole gene deletions of *GPD1L* or *SCN5A* has not been reported.
 - Heterozygous mutations in the tumor suppressor gene *MLH1* cause Hereditary Non-Polyposis Colorectal Cancer syndrome (Lynch syndrome). Therefore, this patient was also given a diagnosis of Lynch syndrome, which causes an increased risk for adult-onset cancers including colorectal, uterine, ovarian, stomach, kidney, urinary tract, small bowel, brain/ central nervous system,

and skin cancers. These malignancies are thought to develop as a result of one germline mutation and one somatic mutation in *MLH1*. This was an incidental finding, not related to this patient's CHD.

- Several genes in the region of interest are associated with autosomal recessive conditions. Homozygous mutations in *SCN5A* are associated with Sick Sinus Syndrome 1 and susceptibility to SIDS (autosomal recessive or multifactorial), homozygous mutations in *GLBI* cause GMI-gangliosidosis and Mucopolysaccharidosis Type IVB, homozygous mutations in *CRTAP* cause Osteogenesis Imperfecta Type IIB and VII, and homozygous mutations in *MYD88* are associated with recurrent pyogenic bacterial infections (60).

Case 110 is an asymptomatic carrier of each of these conditions, and would not be affected except in the case of a mutation in the remaining allele.

- Parental studies: Parental studies were offered because of the implications of Lynch syndrome and other syndromes involving the sodium channel of the heart, as well as to provide reproductive risk information. The parental studies have not yet been completed, therefore it is uncertain if this deletion is de novo or inherited.

In summary, this patient has CHD and minimal dysmorphology with a clinically significant deletion detected by array CGH. This is a large deletion with more than twenty genes in the deleted region (a threshold proposed by Thienpont et al) (12). Because several of these genes have been associated with defects in cardiac structure and function, this deletion is likely causal for the patient's CHD. Of note, this patient was the only patient in our study with a CNV large enough to be detected by both classical cytogenetic analysis and array CGH.

4.1.8 Case 118

Case 118 is a male of unspecified race, born prematurely at 33 weeks gestation. He was diagnosed with a complex CHD including D-TGA with VSD, AS, COA, and DORV. In addition, an EKG showed left ventricular hypertrophy but a normal sinus rhythm. This patient had various extracardiac abnormalities, including a right inguinal hernia, pulmonary insufficiency, right optic nerve hypoplasia, nystagmus, tremors, and feeding intolerance. He received a Medical Genetics consult due to concern for dysmorphic features. Features noted on exam included a sloping and hairy forehead, puffy eyes, very high palate, hypertrophic gums, slight redundant folds of the neck, some joint restriction, fine tremor and hypertonia. There were also exposures during pregnancy that included benzoate, tobacco, and a history of HSV and Chlamydia infection. A brain MRI identified periventricular leukomalacia of the left anterior periventricular white matter, a thin corpus callosum, and delayed myelination, with no evidence of hemorrhage. An ultrasound of the head revealed small bilateral subependymal hemorrhages and a tiny choroid plexus cyst. In addition, a renal ultrasound revealed mild bilateral pelviectasis.

This patient had a normal karyotype by standard cytogenetics, but a duplication in the region of 16q21 of approximately 683 kb was detected by array CGH. Factors considered in the assessment of causality of this CNV included:

- Previous reports: There are no published case reports of similar duplications, and this duplication is not listed in a database of normal variants (55, 57).
- Gene Content: There are a total of seven genes in this region; two are listed in OMIM and both are protein-coding (56). *CNOT1* encodes a subunit of the CCR4-NOT

transcription complex, which is a global regulator of RNA polymerase II transcription (62). *GOT2* encodes mitochondrial glutamate oxaloacetate transaminase, which is an aminotransferase localized to the mitochondria. This protein may be involved in the activity of tyrosine aminotransferase (TAT) in the mitochondria (63). The potential dosage effect of these protein products is unclear; neither genes have been associated with monogenic diseases or with cardiac development (60).

- Parental studies: The duplication was confirmed by FISH to be maternally inherited. This decreases the likelihood that this CNV is causal for the patient's phenotype, as his mother was not found to have similar clinical features, but does not rule out causality due to the possibility of reduced penetrance and variable expressivity. It was recommended that the patient's mother receive a baseline Cardiology evaluation in the case that this duplication is associated with the patient's CHD.

In summary, this patient has a relatively small duplication with unclear clinical significance detected by array CGH; is likely to be benign as the two OMIM genes in the region do not have a known dosage effect and the CNV is inherited. However, this patient has apparently syndromic CHD. The conclusion of a Medical Genetics evaluation was that this duplication does not explain the patient's heart defect and other clinical findings. Testing for other single gene disorders was initiated with *MLL2* for Kabuki syndrome which was negative. A Noonan syndrome panel, urine organic acids for inborn errors of metabolism, and a sterol panel for Smith-Lemli-Opitz syndrome were recommended but not yet completed. Other differentials which may be explored in the future include CHARGE syndrome, DiGeorge syndrome II, and connective tissue disorders.

4.1.9 Case 119

Case 119 is Caucasian male born full-term at 39 weeks gestation and diagnosed with congenital complete heart block and two small ASDs. Growth percentiles at 17 hours of life were 20% for weight, 5% for length, and 13% for head circumference. Features noted on an exam for dysmorphology included hypotelorism, puffy eyes, nails which were extremely thin on the hands and hypoplastic and thin on the feet, edema, jaundice, a dystrophic scar, short neck with redundant skin, ears described as punched out helix and angulated, an extra crease on the hands, and a deep sacral dimple. Pertinent environmental factors in this case included possible enteric virus, sepsis, and maternal lupus. A head ultrasound was normal, while a renal ultrasound revealed mild pelviectasis of the left kidney.

Array CGH revealed a deletion of 3p26.3, spanning at least 295 kb. Factors considered in the assessment of clinical significance of this CNV included:

- Previous Reports: There are no published case reports of similar deletions. This deletion has not been reported in a database of normal variants (55, 57).
- Gene Content: One gene, *CNTN6*, also known as *NB-3*, is located within the deleted region (56). This gene encodes a neural adhesion molecule in the contactin subgroup of the immunoglobulin superfamily. Contactins are expressed exclusively in the nervous system, and are essential to formation and maintenance of synaptic connections in the adult brain, with highest expression in the cerebellum (64). Dosage sensitivity of *CNTN6* is unclear and it has not been associated with any monogenic diseases.
- Parental studies have not yet been completed.

In summary, this patient has a deletion with unclear clinical significance; it is relatively small and includes only one gene which is not known to be associated with cardiac development or monogenic diseases. Without any further information, it appears to be unlikely to be causal for the patient's CHD. This patient has apparently syndromic CHD based on the criteria used for this study, but did not receive a comprehensive evaluation by Medical Genetics to determine if additional genetic testing is warranted.

4.1.10 Case 121

Case 121 is a full-term Caucasian female born at 39 weeks gestation and diagnosed with hypoplastic left heart syndrome, including mitral atresia, hypoplastic left ventricle, aortic atresia, hypoplastic ascending aorta. This patient was small for gestational age with growth percentiles at 1 day of life of 13% for weight, <1% for length, and 15% for head circumference. A basic dysmorphism exam was completed per standard of care, but this patient did not receive a comprehensive Medical Genetics evaluation. Phenotypic features noted on exam included left hip dysplasia, gross lower extremity and labial edema, ankyloglossia, borderline low set ears, possible anteriorly placed anus, prominent chest, hypotonia, and a premature for age appearance.

This patient was found to have a duplication in the region of 1q21.1, spanning at least 1.19 Mb. Factors considered in the assessment of clinical significance included the following:

- Previous reports: Duplications in this region are associated with chromosome 1q21.1 duplication syndrome, which has been well-defined in the literature. This region is rich in complex low-copy repeat segments, and CNVs in the region are frequently inherited and have incomplete penetrance and variable expressivity. Associated features include

macrocephaly, frontal bossing, hypertelorism, hypoplasia of the corpus callosum and cerebellar vermis, seizures, developmental delay and mental retardation, learning disabilities, as well as psychiatric manifestations such as autism and schizophrenia, and various dysmorphic features with no clear pattern (58, 65, 66). Macrocephaly has been reported in 50% of cases of 1q21.1 duplication syndrome, and is thought to be caused by a dosage effect of the *HYDIN* gene located in this region (65). Patient 121 had a head circumference percentile of 15% at 24 hours, so she did not have macrocephaly at the time of assessment. Deletions within the 1q21.1 region have been associated with CHD, particularly anomalies of the aortic arch (67). Case reports of CHD associated with duplications of this region also exist: among 24 patients with a 1q21.1 duplication, Brunetti-Pierri et al. reported one case with a complex CHD. Of note, they also reported one case with congenital hip dysplasia, similar to our patient (58).

- Gene content: This region contains 9 OMIM genes, all of which are protein-coding (56). Two genes are associated with monogenic diseases caused by heterozygous mutations: *GJA5* encodes a connexin protein which is involved in gap junctions for ion and small molecule transport between cells; heterozygous mutations are associated with Familial Atrial Fibrillation-11 (68). *GJA8* encodes a connexin protein which is involved in gap junctions connecting fiber cells of the lens; heterozygous mutations are associated with cataract and microcornea syndromes (69). Because Case 121 has a duplication of this region, not a deletion, these syndromes are not likely to be clinically relevant.
- Parental studies were not completed.

Because this duplication is part of a well-defined syndrome, it is likely that the duplication accounts for this patient's syndromic features. This also provides important

information regarding the monitoring of this patient for any neurologic features. Because CHD has been previously associated with a 1q21.1 duplication, it is likely that this patient's CHD is related to the duplication.

4.1.11 Case 127

Case 127 is a Caucasian female born full-term at 39 and 1/7 weeks gestation. She presented with acute enterovirus myocarditis and severe cardiomyopathy. She was originally suspected to have COA, but this was ruled out; the only structural heart defect remaining was a bidirectional PFO. Growth percentiles at 6 days of life were 67% for weight, 39% for length, and 36% for head circumference. Subtle dysmorphic features were noted on a limited exam, including a microcephalic and plagiocephalic head shape, slight mid facial hypoplasia, deep-set and upslanted eyes, low-set posterior hairline, short neck, shield chest, and deep sacral cleft. An EKG revealed several abnormalities, including a sinus rhythm with occasional premature ventricular complexes, biatrial enlargement, a northwest axis, and a possible lateral infarct. A head ultrasound was normal, while a renal ultrasound revealed a small amount of free perihepatic and pelvic fluid but was normal overall. Of note, this patient's mother had gestational diabetes treated with insulin during the pregnancy and was also treated for hypothyroidism.

Classical cytogenetic analysis was normal but array CGH revealed a deletion of 358 kb in size in the region of chromosome 2q21.3. Factors considered in the assessment of clinical significance included:

- Previous reports: There are no case reports of similar deletions, nor is this deletion found in a database of normal variants (55, 57).

- Gene Content: This region contains two OMIM genes, *CCNT2* and *RAB3GAP1*, as well as three other genes (56). *CCNT2* encodes a cyclin protein which forms a complex with CDK9 which may moderate RNA polymerase II activity (70). *RAB3GAP1* encodes the catalytic subunit of the RAB3 GTPase-activating protein; this protein inactivates RAB3-GTP to regulate synaptic transmission and plasticity (71, 72). Overall, members of the RAB3 family are involved in regulating the exocytosis of neurotransmitters and hormones. Homozygous mutations in *RAB3GAP1* cause Warburg micro syndrome 1. This syndrome is associated with ocular, neurodevelopmental, and metabolic manifestations, specifically hypothalamic hypogonadism (73). Case 127 would be an asymptomatic carrier for this condition, except in the case of a mutation in the remaining allele. Dosage effects of the genes in this region of copy number variation are unclear, and neither OMIM gene is known to be associated with monogenic diseases or cardiac development.
- Parental testing has not yet been completed.

In summary, this patient had severe cardiomyopathy and PFO as well as subtle dysmorphic features. The deletion detected by array CGH is relatively small and does not include any genes implicated in cardiac development. Without further information from published case reports or parental studies, it is unclear whether the deletion detected by array CGH contributed to this patient's CHD and cardiomyopathy. This patient did receive an inpatient Genetics evaluation; an ophthalmology evaluation was recommended to rule out Warburg syndrome, but no additional genetic testing has been completed to date.

4.1.12 Case 128

Case 128 is a Caucasian female born at 38 and 6/7 weeks gestation. Growth percentiles at 15 hours of life were 4% for weight, 12% for length, and 0% for head circumference, consistent with microcephaly. This patient was diagnosed with TOF, including pulmonary atresia and VSD. Abnormal features noted on an exam for dysmorphology included a V-shaped high cleft palate, low-set and posteriorly rotated ears, and a narrow and possibly beaked nose. On examination, other features which could be normal variation included mild plagiocephaly, bitemporal narrowing, low posterior hairline, normal eyes with mild hypertelorism, ankyloglossia, and a sacral dimple. Other extracardiac features included a swallowing disorder and post-surgical thrombosis. Testing confirmed that this patient carries a heterozygous Factor V Leiden mutation which could related to the thrombosis. A head ultrasound was normal, and a renal ultrasound revealed minimal left caliectasis but was otherwise normal. Classical cytogenetic analysis was normal but array CGH revealed a pathogenic deletion spanning 535 kb at chromosome 16p11.2. Factors considered in assessing clinical significance included:

- Previous Reports: This deletion is known as 16p11.2 microdeletion syndrome, a contiguous gene syndrome associated with deletions of approximately 550 kb at this locus. This syndrome is associated with up to 1% of cases of autism spectrum disorder. This syndrome is associated with a variety of other clinical features, including DD/ID, severe early-onset obesity, dysmorphic facial features, congenital anomalies, and possibly other primary psychiatric disorders (74-76). There is also a case report of two monozygotic twins with an aortic valve abnormality, seizure disorder, and mild ID associated with a 16p11.2 microdeletion (59). This syndrome is noted to have variable

expressivity, incomplete penetrance and it can be inherited from either an affected or asymptomatic parent. Males appear to be more likely to be affected with autism than females, even among individuals with this syndrome. Duplications of this region are also associated with autism, but are more likely to have reduced penetrance and less likely to be associated with dysmorphism (74).

- Gene Content: This region contains 18 OMIM genes, including *SPN*, *QPRT*, *MAZ*, *C16orf53*, *MVP*, *CDIPT*, *KCTD13*, *CORO1A*, *BOLA2*, *SULT1A3*, *TAOK2*, *HIRIP3*, *DOC2A*, *ALDOA*, *PPP4C*, *TBX6*, *YPEL3*, and *MAPK3* (56). All are protein-coding, but only one is associated with a monogenic disease. Homozygous mutations in *ALDOA*, which encodes the protein Aldolase A, Fructose-Bisphosphate, cause glycogen storage disease type XII (77). Due to the deletion of this region, Case 128 would be an asymptomatic carrier for this condition, except in the rare case of a mutation in the other allele.
- Parental studies have not yet been completed.

In summary, this patient has an apparently syndromic congenital heart defect. She has a clinically significant deletion of 16p11.2, associated with a well-defined microdeletion syndrome. This syndrome has been associated with multiple congenital anomalies and neurologic manifestations, and therefore likely explains this patient's phenotypic features. Based on previous case reports of CHD associated with 16p11.2 deletions, this deletion is likely causal for the patient's CHD. This patient was evaluated by Medical Genetics, and it was not felt that additional testing was necessary at this time.

4.1.13 Case 132

Patient 132 is a female of unspecified race, born at 39 and 3/7 weeks gestation. This patient was small for gestational age, with growth percentiles at 8 hours of life of 18% for weight, <1% for length, and 1% for head circumference. This patient was diagnosed with a complex CHD, with a functional single ventricle consisting of a double inlet left ventricle and hypoplastic right ventricle, as well as PS and VSD. Features noted on a dysmorphology exam included microcephaly, simple ears, sacral dimple, deep, upslanted eyes, broad-tipped nose, short neck, possibly abnormal nails, weak cry, bicycling movements, and multiple hemangiomas. These capillary malformations appeared as pink macules on the abdomen, back, groin, and genital/ buttock region. Head and renal ultrasounds were normal. Nonspecific debris in the bladder and echogenic renal cortices was noted on the abdominal ultrasound; however this was noted to be possibly normal variation. Other extracardiac abnormalities included left-sided hemihypertrophy and a portal vein thrombus.

This patient had a normal karyotype by classical cytogenetic analysis, but a 346 kb deletion at chromosome 22q12.3 was detected by array CGH. Factors considered in the assessment of pathogenicity included the following:

- Previous Reports: Similar deletions have not been previously reported, and this CNV has not been reported in a database of healthy individuals (55, 57).
- Gene Content: This deletion contains a portion of the gene *LARGE* (56) , which is a member of the N-acetylglucosaminyltransferase family. *LARGE* recognition and glycosylation is essential to the expression of functional alpha-dystroglycan which prevents muscle degradation (78, 79). Homozygous mutations or deletions of this gene

have been associated with Walker-Warburg syndrome, also known as muscular dystrophy-dystroglycanopathy types A and B. Type A includes muscular dystrophy with brain anomalies and eye anomalies, while type B includes muscular dystrophy and mental retardation; onset ranges from prenatal to six months of life (80, 81). Patient 132 would be an asymptomatic carrier for these syndromes unless she carried a mutation in the remaining copy of *LARGE*. An elevated CPK level would be expected in patients with these syndromes; this patient's CPK was 37 (reference range of 42-470).

- Parental studies: This deletion was determined to be paternal in origin by FISH analysis.

In summary, Patient 132 has an apparently syndromic CHD, with various extracardiac abnormalities and dysmorphic features. The deletion detected by array CGH has unclear clinical significance. It is less likely to be causal for this patient's phenotype because it was also detected in the patient's father, but this does not rule out the possibility of pathogenicity due to variable expressivity. This patient did receive a comprehensive Medical Genetics evaluation. Due to this patient's hemihypertrophy and capillary malformations, Klippel Tranauny Weber syndrome was considered as a differential diagnosis.

4.1.14 Case 140

Patient 140 is a Caucasian female born at 38 and 3/7 weeks gestation. This patient was small for gestational age; growth percentiles at 30 hours of life were <1% for weight, length, and head circumference. This patient was diagnosed with an unbalanced complete AVSD, with a dominant right ventricle, small left ventricle, and transverse arch hypoplasia (causing right ventricular outflow tract obstruction). This CHD was apparently isolated: no dysmorphic

features were noted on exam and the patient was considered non-dysmorphic in appearance both by Cardiology and Genetics evaluations. Of note, this patient did have sclera erythema which was attributed to labor, as well as symptoms of neonatal narcotic withdrawal from a maternal history of hepatitis C and heroin use, smoking and methadone dependency.

This patient had a normal karyotype by classical cytogenetic analysis but a deletion of 1.36 Mb was detected by array CGH. Factors considered in the assessment of clinical significance included the following:

- Previous reports: Similar deletions have not been reported in the literature or in a database of healthy controls (55, 57).
- Gene content: The deleted region contains the 5' region of *SUCLG2* and is just upstream of *TMF2*, both of which are OMIM genes (55). *SUCLG2* encodes the beta subunit of Succinate-CoA ligase; this enzyme is localized to the mitochondria and is involved in the tricarboxylic acid cycle and carbohydrate metabolism (82). *TMF2*, or TATA element modulatory factor 1, has several functions. It mediates the degradation of STAT3 (involved in the cell response to interleukins), possibly acts as a coactivator of the androgen receptor, and binds the TATA element of HIV-1 to inhibit transcription (82). The deleted region also contains two other protein-coding genes not listed in OMIM, *FAM19A1* and *FAM19A4*, which may be involved in regulation of immune and nervous cells in the brain (83). None of these genes have been associated with monogenic diseases, but because of their roles in metabolism and transcriptional regulation, a dosage effect is possible.
- Parental studies: Parental studies have not yet been completed.

This deletion has unclear clinical significance, as there is a paucity of information from previous reports or parental studies to assess pathogenicity. In addition, it is possible that maternal environmental exposures contributed to this patient's heart defect. Because this patient had apparently isolated CHD, she did not receive a full Medical Genetics evaluation and no additional testing for single gene disorders was completed.

4.1.15 Case 141

Patient 141 is a male with unknown race born at 39 weeks gestation. Growth percentiles at 21 hours were 2.91% for weight, 25.26% for length, and 5.76% for head circumference; this patient had symmetric IUGR. This patient was diagnosed with hypoplastic left heart syndrome (HLHS), including mitral stenosis, aortic atresia, and a small secundum ASD. Several abnormal features were noted on a dysmorphology examination, including deep-set and puffy eyes, down-slanting palpebral fissures, a loose, webbed and short neck, a deep sacral dimple, right club foot, and loose skin with gross edema. Other phenotypic features which may be normal variation included a frontal bossing, low set and posteriorly rotated ears with an over-folded right ear and a Darwinian tubercle of the left ear, a broad nasal bridge, hypoplastic nipples, slight shield chest appearance, 5th left finger clinodactyly, hypoplastic toenails, and a decreased suck reflex. Additional extracardiac features included edema, presumed sepsis, and a diagnosis of congenital hypothyroidism. A head ultrasound was normal and a renal ultrasound revealed minimal left upper pole caliectasis but was otherwise normal. Due to the deep sacral dimple, a spine ultrasound was ordered to rule out a tethered cord, which was normal. This patient was noted to

be non-dysmorphic by Cardiology but was considered to have an overall dysmorphic appearance by Medical Genetics.

This patient had a normal karyotype by classical cytogenetic analysis, but array CGH detected a duplication in the region of chromosome 20p11.21 spanning approximately 398 kb (breakpoints 25,016,046- 25,414,542). Factors considered in the assessment of clinical significance included the following:

- Previous Reports: Similar duplications have not been reported in a database of healthy individuals. One report of an individual with a similar duplication (breakpoints 25,096,493- 25,467,359) inherited from a normal parent was noted to have dysmorphic features, high palate, joint laxity, tall stature, DD/MR, voice abnormalities and behavioral problems (55, 57).
- Gene Content: The duplicated region contains 5 OMIM genes, including *ENTPD6*, *PYGB*, *ABHD12*, *GINS1*, and *NINL* (56). *ENTPD6* encodes an enzyme which may be involved in the glycosylation reactions in the Golgi apparatus and the catabolism of extracellular nucleotides; it is expressed mainly in the heart. *PYGB* encodes a glycogen phosphorylase which is involved in carbohydrate metabolism. *ABHD12* encodes a lipase which is involved in synaptic plasticity and neuroinflammation (82). Homozygous mutations in this gene cause a syndrome involving polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC) (84); however, this patient carries a duplication of the *ABHD12* gene. *GINS1* encodes a subunit of the GINS complex which is involved in DNA replication. *NINL* encodes a protein which is involved in microtubule organization of interphase cells (82).
- Parental studies have not been completed.

In summary, this patient had apparently syndromic CHD and a relatively small duplication with unclear clinical significance. There was one report of a similar duplication which was pathogenic, but not associated with CHD. It is unclear whether this CNV contributed to this patient's CHD. This patient received an inpatient genetics consult prior to receiving results from array CGH. Because of the constellation of dysmorphic features and extracardiac features in this patient, testing for Noonan syndrome was ordered and was pending at the time of this report.

4.1.16 Case 102

Patient 102 is a female of unspecified race, born at 39 weeks gestational age. This patient was small for gestational age, with growth percentiles at 10 days of life of 23% for weight and <1% for length and head circumference. This patient was diagnosed with Tetralogy of Fallot. Phenotypic features noted on a Medical Genetics examination included a large anterior fontanelle, bell-shaped chest, prominent calcaneus (heel bone) of the feet bilaterally, a crease in the right arm similar to Madelung's deformity, pauses in breathing, and somewhat hypoplastic nails. Extracardiac features included subglottic stenosis requiring a tracheostomy and a humerus fracture. However, bones and soft tissue were otherwise normal, as were a brain MRI, head ultrasound and renal ultrasound. An abdominal x-ray showed trace ascites.

Classical cytogenetic analysis revealed a balanced translocation which was paternally inherited; the karyotype was 46,XX,t(6:10)(p21.2;p15). Normal array CGH results confirmed the balanced nature of this translocation at a resolution of 135 kb. This patient did receive a complete Genetics evaluation. Because this patient had multiple congenital anomalies, there was

strong suspicion for a genetic syndrome. Because the translocation was confirmed to be balanced, it is not likely to be causal for this patient's phenotype unless imprinted genes in the region are affected. In addition, the patient's father, who also carries the translocation, is unaffected. Of note, the DiGeorge syndrome II region is located at 10p13-14 and the breakpoint for this patient's translocation is at 10p15; the significance of this proximity to the DiGeorge region is unclear because there is no apparent loss or gain of genetic material.

To this point, no additional genetic testing has been performed, but the suggested differentials include a microdeletion syndrome not detected by this array CGH platform, single gene disorders such as Noonan syndrome, Kabuki syndrome, Alagille syndrome, or a single gene mutation in *JAGGED1* or *NKX2.5*. The patient will be monitored for features which would be suggestive of any specific syndromes and further testing may be performed in the future.

APPENDIX B: IRB APPROVAL LETTERS



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu>

Memorandum

To: Suneeta Madan-Khetarpal MD
From: Sue Beers PHD, Vice Chair
Date: 4/4/2011
IRB#: PRO11010510
Subject: Chromosome Abnormalities in Neonates with Congenital Heart Defects

The University of Pittsburgh Institutional Review Board reviewed and approved the above referenced study by the expedited review procedure authorized under 45 CFR 46.110 and 21 CFR 56.110. Your research study was approved under:
45 CFR 46.110.(5) clinical data

Approval Date: 4/4/2011
Expiration Date: 4/3/2012

For studies being conducted in UPMC facilities, no clinical activities can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.



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Institutional Review Board

3500 Fifth Avenue
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Memorandum

To: Suneeta Madan-Khetarpal MD
From: Sue Beers PHD, Vice Chair
Date: 2/2/2012
IRB#: REN12010228 / PRO11010510
Subject: Chromosome Abnormalities in Neonates with Congenital Heart Defects

Your renewal for the above referenced research study has received expedited review and approval from the Institutional Review Board under:
45 CFR 46.110.(5) clinical data

Please note the following information:

Approval Date: 2/1/2012
Expiration Date: 1/31/2013

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

BIBLIOGRAPHY

1. Hoffman J. Incidence of congenital heart disease: I. Postnatal incidence. *Pediatr Cardiol.* 1995;**16**:103-13.
2. Nora JJ. Causes of congenital heart diseases: old and new modes, mechanisms, and models. *Am Heart J.* 1993;**125**:1409-19.
3. Ferencz C, Neill CA, Boughman JA, Rubin JD, Brenner JI, Perry LW. Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study. *J Pediatr.* 1989;**114**:79-86.
4. Pierpont ME, Basson CT, Benson W Jr., Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, and Webb CL. Genetic basis for congenital heart defects: Current knowledge: A scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiac Disease in the Young. *Circulation.* 2007;**115**:3015-38.
5. Woodrow Benson D, Sharkey A, Fatkin D, Lang P, Basson CT, McDonough B, Strauss AW, Seidman JG, Seidman CE. Reduced penetrance, variable expressivity, and genetic heterogeneity of familial atrial septal defects. *Circulation.* 1998;**97**:2043-8.
6. Hoffman JI, Kaplan S. The incidence of congenital heart disease. *J Am Coll Cardiol.* 2002;**39**:1890-990.
7. Bernstein D. Evaluation of the cardiovascular system. In: *Nelson Textbook of Pediatrics.* Behrman RE, Kliegman RM, Jenson HB, editor. 17 ed. Philadelphia: Saunders; 2004. p. 1481-8.
8. Mitchell ME, Sander TL, Klinkner DB, and Tomita-Mitchell A. The molecular basis of congenital heart disease. *Semin Thorac Cardiovasc Surg.* 2007;**19**:228-37.
9. Hartman RJ, Rasmussen SA, Botto LD, Riehle-Colarusso R, Martin CL, Cragan JD, Shin M, Correa A. The contribution of chromosomal abnormalities to congenital heart defects: a population based study. *Pediatr Cardiol.* 2011;**32**:1147-57.
10. Johnson MC, Hing A, Wood MK, Watson MS. Chromosome abnormalities in congenital heart disease. *Am J Med Genet.* 1997;**70**:292-8.

11. Richards AA, Santos LJ, Nichols HA, Crider BP, Elder FF, Hauser NS, Zinn AR, and Garg V. Cryptic chromosomal abnormalities identified in children with congenital heart disease. *Ped Res.* 2008;**64**:358-63.
12. Thienpont B, Mertens L, de Ravel T, Eyskens B, Boshoff D, Mass N, Fryns J-P, Gewillig M, Vermeesch JR, and Devriendt K. Submicroscopic chromosomal imbalances detected by array-CGH are a frequent cause of congenital heart defects in selected patients. *Eur Heart J.* 2007;**28**:2778-84.
13. Beauchesne LM, Warnes CA, Connolly HM, Ammash NM, Grogan M, Jalal SM, Michels VV. Prevalence and clinical manifestations of 22q11.2 microdeletion in adults with selected conotruncal anomalies. *J Am Coll Cardiol.* 2005;**45**(4):595-8.
14. Lammer EJ, Chak JS, Iovannisci DM, Schultz K, Oseogawa K, Yang W, Carmichael SL, and Shaw GM. Chromosomal abnormalities among children born with conotruncal cardiac defects. *Birth Defects Res A Clin Mol Teratol.* 2009;**85**:30-5.
15. Lu X-Y, Phung MT, Shaw CA, Pham K, Neil SE, Patel A, Sahoo T, Bacino CA, Stankiewicz P, Kang S-HL, Lalani S, Chinault AC, Lupski JR, Cheung SW, Beaudet AL. Genomic imbalances in neonates with birth defects: High detection rates by using chromosomal microarray analysis. *Pediatr* 2008;**122**:1310-8.
16. Miller DT, Adam MP, Aradhya S, Blesecker LG, Brothman AR, Carter NP, Church DM, Crolla JA, Eichler EE, Epstein CJ, Faucett WA, Feuk L, Friedman JM, Hamosh A, Jackson L, Kaminsky EB, Kok K, Krantz ID, Kuhn RM, Lee C, Ostell JM, Rosenberg C, Scherer SW, Spinner NB, Stavropoulos DJ, Tepperberg JH, Thorland EC, Vermeesch JR, Waggoner DJ, Watson MS, Martin CL, and Ledbetter DH. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet.* 2010;**86**:749-64.
17. Kodo K, Yamagishi H. A decade of advances in the molecular embryology and genetics underlying congenital heart defects. *Circ J.* 2011;**75**:2296-304.
18. Botto LD, Lin A, Riehle-Colarusso T, Malik S, Correa A, The National Birth Defects Prevention Study. Seeking Causes: Classifying and Evaluating Congenital Heart Defects in Etiologic Studies. *Birth Defects Research Part A.* 2007;**79**:714-27.
19. Hypoplastic Left Heart Syndrome [Diagram]. Lucile Packard Children's Hospital at Stanford. 2012 [cited March 28, 2012]; Available from: <http://www.lpch.org/DiseaseHealthInfo/HealthLibrary/cardiac/hlhs.html>.
20. Kliegman RM, Stanton BF, St. Geme JW, Schor NF, and Behrman RE, editor. *Nelson Textbook of Pediatrics.* 19th ed. Philadelphia: Elsevier Saunders 2011.

21. Nora JJ, Nora AH, Toews WH. Letter: Lithium, Ebstein's anomaly, and other congenital heart defects. *Lancet*. 1974;**2**(7880):594-5.
22. Williams WG MB, Caldarone C, Cullen-Dean G, Cai S, Levesque O, Cumberbatch C. Summary of the Interrupted Aortic Arch (IAA) Analysis: A Congenital Heart Surgeons Society Study. The Hospital for Sick Children: Congenital Heart Surgeons' Society Data Center; 2004.
23. Schumacher KR, Zieve D. Endocardial cushion defect. March 21, 2012 [cited March 30, 2012]; Available from: <http://www.nlm.nih.gov/medlineplus/ency/article/007324.htm>.
24. Vergara P, Digilio MC, Zorzi AD, Carlo DD, Capolino R, Rimini A, Pelegri M, Calabro R, Marino B. Genetic heterogeneity and phenotypic anomalies in children with atrioventricular canal defect and tetralogy of Fallot. *Clin Dysmorph*. 2006;**15**:65-70.
25. Debrus S, Berger G, de Meeus A, Sauer U, Guillaumont S, Voisin M, Bozio A, Demczuk S, Aurias A, Bouvagnet P. Familial non-syndromic conotruncal defects are not associated with a 22q11 microdeletion. *Hum Genet*. 1996;**97**(2):138.
26. Congenital Heart Defects. Centers for Disease Control and Prevention. March 17, 2011 [cited March 30, 2012]; Available from: <http://www.cdc.gov/ncbddd/heartdefects/>.
27. Gill HK, Splitt M, Sharland GK, Simpson JM. Patterns of recurrence of congenital heart disease. *J Am Coll Cardiol*. 2003;**42**(5):923-9.
28. Øyen N, Poulsen G, Boyd HA, Wohlfahrt J, Jensen PKA, Melbye M. Recurrence of congenital heart defects in families. *Circulation*. 2009;**120**:295-301.
29. Øyen N, Poulsen G, Wohlfahrt J, Boyd HA, Jensen PKA, Melbye M. Recurrence of discordant congenital heart defects in families. *Circ Cardiovas Genet*. 2010;**3**:122-8.
30. Erdogan F, Larsen LA, Zhang L, Tümer Z, Tommerup N, Chen W, Jacobsen JR, Schubert M, Jurkatis J, Tzschach A, Ropers H-H, Ullmann R. High frequency of submicroscopic genomic aberrations detected by tiling path array comparative genome hybridisation in patients with isolated congenital heart disease. *J Med Genet*. 2008;**45**:704-9.
31. Snijder CA, Vlot IJ, Burdorf A, Obermann-Borst SA, Helbing WA, Wildhagen MF, Steegers EA, Steegers-Theunissen RP. Congenital heart defects and parental exposure to chemicals. *Hum Reprod*.
32. McDermott DA, Fong JC, Basson CT. Holt-Oram Syndrome. In *Gene Reviews*: NCBI, Updated January 4, 2011.
33. Kniffin CL, Jackson JF. Noonan Syndrome [#163950]. Online Mendelian Inheritance in Man; Updated October 26, 2010.

34. Kniffin CL, Jackson JF. Alagille Syndrome [#118450]. Online Mendelian Inheritance in Man; Updated February 9, 2012.
35. Spinner NB, Hutchinson AL, Krantz ID, Kamath BM. Alagille Syndrome. In *Gene Reviews*: NCBI, Updated: July 20, 2010.
36. McDonald-McGinn DM, Emanuel BS, Zackai EH. 22q11.2 Deletion Syndrome. In *Gene Reviews*: NCBI. Updated December 16, 2005.
37. Trask BJ. Human cytogenetics: 46 chromosomes, 46 years and counting. *Nat Rev Genet*. 2002;**3**:769-78.
38. Nora JJ, Berg K, Nora AH. Cardiovascular Diseases: Genetics, Epidemiology, and Prevention. New York, Oxford: Oxford University Press; 1991.
39. Anderlid BM, Schoumans J, Anneren G, Sahlen S, Kyllerman M, Vujic M, Hagberg B, Blennow E, Nordenskjold M. Subtelomeric rearrangements detected in patients with idiopathic mental retardation. *Am J Med Genet*. 2002;**107**:275-84.
40. Baker E, Hinton L, Callen DF, Aitree M, Dobbie A, Eyre HJ, Sutherland GR, Thompson E, Thompson P, Woollatt E, Haan E. Study of 250 children with idiopathic mental retardation reveals nine cryptic and diverse subtelomeric chromosome anomalies. *Am J Med Genet*. 2002;**107**:285-93.
41. Schellberg R, Schwanitz G, Gravinghoff L, Kallenberg R, Trost D, Raff R, Wiebe W. New trends in chromosomal investigation in children with cardiovascular malformations. *Cardiol Young*. 2004;**14**:622-9.
42. Momma K. Cardiovascular anomalies associated with chromosome 22q11.2 deletion syndrome. *Am J Cardiol*. 2010;**105**:1617-24.
43. Baker K, Sanchez-de-Toledo J, Munoz R, Orr R, Kiray S, Shiderly D, Clemens M, Wearden P, Morell VO, Chrysostomou C. Critical congenital heart disease- utility of routine screening for chromosomal and other extracardiac anomalies *Congenit Heart Dis*. 2012;**7**:145-50.
44. Beaudet AL, Belmont JW. Array-Based DNA Diagnostics: Let the Revolution Begin. *Annu Rev Med* 2008;**59**:113-29.
45. Breckpot J, Thienpont B, Peeters H, de Ravel T, Singer A, Rayyan M, Allegaert K, Vanhole C, Eyskens B, Vermeesch JR, Gewillig M, Devriendt K. Array comparative genomic hybridization as a diagnostic tool for syndromic heart defects. *J Pediatr*. 2010;**156**:810-7.

46. Manning M, Hudgins L, Professional Practice and Guidelines Committee. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet Med*. 2012;**12**:742-5.
47. Scott SA, Cohen N, Brandt T, Toruner G, Desnick RJ, Edelmann L. Detection of low-level mosaicism and placental mosaicism by oligonucleotide array comparative genomic hybridization. *Curr Opin Genet Dev*. 2007;**17**:182-92.
48. Position Statements. National Society of Genetic Counselors, [cited March 2012]; Available from: <http://www.nsgc.org/Advocacy/PositionStatements/tabid/107/Default.aspx#PrenatalChildTestingAdultOnset>].
49. Aradhya S, Cherry AM. Array-based comparative genomic hybridization: clinical contexts for targeted and whole-genome designs. *Genetics in Medicine*. 2007;**9**(9):553-9.
50. Redon R, Ishikawa S, Fitch KR, Feuk L et al. . Global variation in copy number in the human genome. *Nature*. 2006;**444**:444-54.
51. Shaffer LG, Bejjani BA, Torchia B, Kirkpatrick S, Coppinger J, Ballif BC. The identification of microdeletion syndromes and other chromosome abnormalities: cytogenetic methods of the past, new technologies for the future. *Am J Med Genet Part C*. 2007;**145C**:335-45.
52. Wincent J, Anderlid B-M, Lagerber M, Nordenskijold M, and Schoumans J. High-resolution molecular karyotyping in patients with developmental delay and/or multiple congenital anomalies in a clinical setting. *Clin Genet*. 2011;**79**:147-57.
53. Sagoo GS, Butterworth AS, Sanderson S, Shaw-Smith C, Higgins JPT, Burton H. Array CGH in patients with learning disability (mental retardation) and congenital anomalies: updated systematic review and meta-analysis of 19 studies and 13,926 subjects. *Genetics in Medicine* 2009;**11**:139-46.
54. Goldmuntz E, Paluru P, Glessner J, Hakonarson H, Biegel JA, White PS, Gai X, and Shaikh TH. Microdeletions and Microduplications in Patients with Congenital Heart Disease and Multiple Congenital Anomalies *Congenital Heart Disease*. 2011;**6**:592-602.
55. DECIPHER v5.1. Wellcome Trust Sanger Institute, [cited March 2012]; Available from: <http://decipher.sanger.ac.uk/>
56. Genoglyphix Genome Browser (Build hg 18 March 2006). PerkinElmer, Genoglyphix, Signature Genomics, [cited March 2012]; Available from: https://www.genoglyphix.com/ggx_browser/search/.
57. Database of Genomic Variants (Build 36 March 2006). The Centre for Applied Genomics, [cited March 2012]; Available from: <http://projects.tcag.ca/variation/>.

58. Brunetti-Pierri N, Berg JS, Scaglia F, Belmont J, Bacino CA, Sahoo T, Lalani SR, Graham B, Lee B, Shinawi M et al. . Recurrent reciprocal 1q21.1 deletions and duplications associated with microcephaly or macrocephaly and developmental and behavioral abnormalities. *Nat Genet* 2008;**40**:1466-71.
59. Ghebranious N, Giampietro PF, Wesbrook FP, Rezkalla SH. A novel microdeletion at 16p11.2 harbors candidate genes for aortic valve development, seizure disorder, and mild mental retardation. *Am J Med Genet A*. 2007;**143A**(13):1462-71.
60. Online Mendelian Inheritance in Man. Johns Hopkins University. 2012, [cited March 2012]; Available from: <http://www.omim.org/>.
61. Liu HX, Oei PTSP, Mitchell EA, McGaughran JM. Interstitial deletion of 3p22.2-p24.2: the first reported case. *J Med Genet*. 2001;**38**:249-351.
62. Albert TK, Lemaire M, van Berkum NL, Gentz R, Collart MA, Timmer HTM. Isolation and characterization of human orthologs of yeast CCR4-NOT complex subunits. *Nucleic Acids Res*. 2000;**28**:809-17.
63. Andersson SM, Pispa J. Purification and properties of human liver tyrosine aminotransferase. *Clin Chim Acta*. 1982;**125**:117-23.
64. Kamei Y, Tsutsumi O, Taketani Y, Watanabe K. *J Neuroscience Res*. 1998;**51**:275-83.
65. Mefford HC, Sharp AJ, Baker C, Itsara A, Jiang Z, Buysse K, Huang S, Maloney VK, Crolla JA, Baralle D, et al. . Recurrent rearrangements of chromosome 1q21.1 and variable pediatric phenotypes. *N Engl J Med*. 2008;**359**:1685-99.
66. Hamosh A. Chromosome 1q21.1 duplication syndrome [#612475]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2011.
67. Christiansen J, Dyck JD, Elyas BG, Lilley M, Bamforth JS, Hicks M, Sprysak KA, Tomaszewski R, Haase SM, Vincen-Wyhony LM, Somerville MJ. Chromosome 1q21.1 contiguous gene deletion is associated with congenital heart disease. *Circ Res*. 2004;**11**:1429-35.
68. O'Neill MJF. Gap junction protein, alpha-5; GJA5 [*121013]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2011.
69. O'Neill MJF. Gap junction protein, alpha-8; GJA8 [+600897]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2008.
70. Nguyen VT, Kiss T, Michels AA, Bensaude O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature*. 2001;**414**:322-5.

71. Alagianis IA, Johnson CA, Gissen P, Chen D, Hampshire D, Hoffmann K, Maina EN, Morgan NV, Tee L, Morton J, Ainsworth JR, Horn D et al. . Mutations of the catalytic subunit of RAB3GAP cause Warburg Micro syndrome. *Nat Genet.* 2005;**37**:221-3.
72. Sakane A, Manabe S, Ishizaki H, Tanaka-Okamoto M, Kiyokage E, Toida K, Yoshida T, Miyoshi J, Kamiya H, Takai Y, Sasaki T. Rab3 GTPase-activation protein regulates synaptic transmission and plasticity through the inactivation of Rab3. *Proc Nat Acad Sci.* 2006;**103**:10029-34.
73. O'Neill MJF. Warburg Micro Syndrome 1; WARBM1 [#600118]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2011.
74. Fernandez BA, Roberts W, Chung B, Weksberg R, Meyn S, Szatmari P, Joseph-George AM, MacKay S, Whitten K, Noble B, Vardy C, Crosbie V, Luscombe S, Tucker E, Turner L, Marshall CR, Scherer SW. Phenotypic spectrum associated with de novo and inherited deletions and duplications at 16p11.2 in individuals ascertained for diagnosis of autism spectrum disorder. *J Med Genet.* 2010;**47**:195-203.
75. Bochukova EG, Huang N, Keogh J, Henning E, Purmann C, Blaszczyk K, Saeed S, Hamilton-Shield J, Clayton-Smith J, O'Rahilly S, Hurles ME, Farooqi IS. Large, rare chromosomal deletions associated with severe early-onset obesity. *Nature.* 2010;**463**:666-70.
76. Walters RG, Jacquemont S, Valsesia A, de Smith AJ, Martinet D, Andersson J, Falchi M, Chen F, Andrieux J, Lobbens S, Delobel B, Stutzmann F et al. . A new highly penetrant form of obesity due to deletions on chromosome 16p11.2. *Nature.* 2010;**463**:671-5.
77. Amberger JS. Aldolase A, Fructose-Bisphosphate; ALDOA [*103850]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2008.
78. Peyrard M, Seroussi E, Sandberg-Nordqvist A-C, Xie Y-G, Han F-Y, Fransson I, Collins J, Dunham I, Kost-Alimova M, Imreh S, Dumanski JP. The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. *Proc Nat Acad Sci.* 1999;**96**:598-603.
79. Kanagawa M, Saito F, Kunz S, Yoshida-Moriguchi T, Barresi R, Kobayashi YM, Muschler J, Dumanski JP, Michele DE, Oldstone MBA, Campbell KP. Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* 2004;**117**:953-64.
80. Kniffen CL. Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 3; MDDGA3 [#253280]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2010.
81. Kniffen CL. Muscular dystrophy-dystroglycanopathy (congenital with mental retardation), type B, 6; MDDGB6 [#608840]. Online Mendelian Inheritance in Man: Johns Hopkins University; 2011.

82. UniProt. UniProt Consortium; 2012, [cited March 2012]; Available from: <http://www.uniprot.org/>.
83. RefSeq Gene. UCSC Bioinformatics; July 2008, [cited March 2012]; Available from: <http://genome.ucsc.edu/index.html>.
84. Fiskerstrand T, H'mida-Ben BD, Johansson S, M'zahem A, Haukanes BI, Drouot N, Zimmerman J, Cole AJ, Vedeler C, Bredrup C, Assoum M, Tazir M, Klockgether T, Hamri A, Steen VM, Boman H, Bindoff LA, Koenig M, Knappskog PM. Mutations in ABHD12 cause the neurodegenerative disease PHARC: an inborn error of endocannabinoid metabolism. *Am J Hum Genet.* 2010;**87**:410-7.