A Study of Birth Defects: Genetic and Epigenetic Determinants

Chapter 1: Introduction
1.1 Origin of proposal

According to the World Health Organization’s (WHO) document of 1972, “congenital malformations” are defined as structural defects present in the infants at birth. However, in the recent WHO fact-sheet (October, 2012), both structural and functional anomalies have been included in this category (Sarkar et al., 2013).

Globally, birth defects or congenital malformations pose a significant health concern and are a leading cause of the infant mortality and morbidity. Worldwide, birth defects account for 3% to 4% of all live births and are a major cause of elective pregnancy terminations or spontaneous abortions (Hobbs et al., 2005). In the United States, birth defects affect 1 in every 33 newborns resulting in long-term disability and leads to mortality in extreme cases (Centers for Disease & Prevention, 2008). In India, the prevalence of birth defects is between 61 and 70 per 1000 live births. Most of the birth defects form at the time of organogenesis, during the 3rd and 8th week of gestation. Birth defects or congenital anomalies may result in either complete or partial lack of an anatomical element or modification of its regular organization. Mostly, these are caused by genetic or environmentally induced factors, acting individually or in concert. An additional 2 to 3% are documented in children below age 5 years (Raza et al., 2012).

Down Syndrome (DS), a consequence of chromosomal aneuploidy, is a prevalent genetic disorder resulting in intellectual disability and several other phenotypic and clinical anomalies. Globally, DS alone accounts for 15-20% of intellectual disability (S. E. Antonarakis, 2017). Trisomy 21 which involve the occurrence of an extra chromosome 21 or part of it instead of two chromosomes is the major known cause of DS. Besides Trisomy 21, there are two other minor categories of DS called translocational and mosaic DS accounting for 2 and 3% of total DS population, respectively (Fisch et al., 2003).

The incidence of DS is 1 in 920 to 1,000 in the general population with average life expectancy of 55 years. In India, around 1 in 920 live births are born with DS while globally the frequency is around 1 in 1,000 (Verma, 2000). There are about 250,000 families in the United States of America with a DS child (“WHO | International Collaborative Research on Craniofacial Anomalies”, 2017).
Congenital Heart Diseases (CHD) comprises a group of major congenital anomalies that has become a global health problem in recent past. The prevalence of CHD varies among studies worldwide, however, it is estimated that CHD is prevalent in approximately 8 per 1000 live births (van der Linde et al., 2011).

The etiology of CHD remains largely unknown. Cardiac development involves a controlled pathway comprising of various developmental pathway molecules, transcription factors, and epigenetic regulators. Mutations in these genes sometimes lead to disruption of normal signaling cascades leading to CHD (Qian et al., 2017). In approximately 15% it was linked with several well-recognized aneuploidies that comprise the CHD. The prevalence of CHD in DS patients is approximately 45% (Benhaourech et al., 2016).

The reported risk factors for CHD include various factors such as the use of certain drugs, e.g. thalidomide, retinoic acid as well as exposure to some organic solvents. Maternal exposure to herbicides and parental age >40 years 50% men and 33% women have also been reported as risk factors for CHD in some reports (Roeters van Lennep et al., 2002). The most common form of CHD is the Tetralogy Of Fallot (TOF). This particular defect is responsible for 7-10% of all the CHD cases and consists of anatomical malfunctions, including Ventricular Septal Defect (VSD), right ventricular hypertrophy, pulmonary stenosis and an prevailing aorta (Bruneau, 2008).

The strong association between DS and CHD has been well studied across the scientific community. It has been shown that both the conditions have many common etiologies and risk factors. The knowledge of these causes and risk factors can provide useful information to modify, manage or even prevent the conditions and the morbidity associated with these diseases.

Therefore, an in depth understanding of the alternative risk factors besides the known major ones is the need of the hour. Hence, this work is aimed at establishing several lifestyle and environmental factors as major influencers in developing Trisomy 21 as well as CHD in newborns.
1.2. Definition of the problem

Congenital anomalies possess a great health burden worldwide. They can be either functional or structural. Most of the structural birth imperfections usually develop in the first 2-8 weeks of the embryogenesis. Birth defects can also be classified as an isolated one, where only one organ system is affected or it can affect multiple organs. Based on the organ systems affected, birth defects can be classified into two main categories:

1.2.1 Structural birth defects

This type of birth defect affects the development of any body part or structure. Examples includes

a) Cleft lip or cleft palate.

b) Congenital heart defects, for example, complete absence or altered shaped valves.

c) Atypical limbs, such as a clubfoot.

d) Neural tube imperfection, such as spina bifida.

1.2.2. Functional birth defects

Functional or developmental birth defects affect the functionality of an organ system. These defects frequently lead to Intellectual and Developmental Disability (IDD) and include:

- **Brain and Nervous system disorders**: These categories comprise IDDs, speech or language difficulties, behavioural disorders, seizures, and movement trouble. Very few affect which affects the nervous include DS.

- **Sensory disorders**: Examples include loss of hearing and some visual difficulty, such as blindness.

- **Metabolic disorders**: This group includes inconvenience with metabolism and excretion of the waste metabolic end products of the body. In such conditions the individual’s body’s is unable to rid of waste materials or damaging chemicals. Two common examples of congenital anomalies related to metabolic disorders are phenylketonuria and hypothyroidism.
• **Degenerative disorders:** These are a group of congenital anomalies that are not found at birth. However, these disorders affect one or more health conditions steadily over a period. For example, muscular dystrophy and X-linked adrenoleukodystrophy are some of the degenerative disorders that lead to problems of the nervous system and the adrenal glands later in life.

Birth defects can be also be classified based on how the condition is severe. The defect that requires surgical intervention and might result in death of the newborn is called major birth defects, whereas, the other type is classified as minor. This minor group is extensively harmful to the quality of life and health of the patient but are never life-threatening. Though, this categorization is not suitably defined, as some minor anomalies can be linked with underlying major defects. This association between the major and minor anomalies could range from 3, 10 and 20% in patients having one, two or more than three anomalies, respectively (Raza et al., 2012). Among these congenital anomalies, some are visible at birth, for e.g. cleft palate; while, a few anomalies such as congenital dislodgment of the hip, may not be detected in the beginning, while others may appear much later in life, for e.g. Patent Ductus Arteriosus (PDA). Internal defects, when they are not fatal, most of the times they go unrecognized. However, in any case, these defects are a key source of morbidity and mortality of infant’s across the globe, and responsible for as many as 260,000 deaths (7% of all infant deaths) in the year 2004 (Raza et al., 2012). Studies have shown that 94% of severe congenital anomalies occur in low and middle-income countries possibly due to lack of nutritious foods, infections and lack of proper healthcare and screening tests.

Nonsyndromic defects may occur when the birth defects are not associated with multi-organ syndrome. The etiologies or the precise cause of the structural birth defects remain unclear. However, it been proposed that most of them are consequence of a complex interaction between genetic, epigenetic, environmental, and lifestyle aspects (Hobbs et al., 2005).

### 1.3 Down Syndrome (DS)

It is one of the key causes of Intellectual Disability (ID) among children across the globe. Besides ID, DS is characterized by several associated co-morbidities such as CHD, leukaemia, learning disabilities, cancers and many more. The disparity of genes located on
human chromosome 21 is known to be the foremost etiologic aspect for this condition. In the majority of the cases an extra copy of chromosome 21 results in a condition called trisomy. The other causes of DS can be Robertsonian translocation and isochromosome or ring chromosome (S. E. Antonarakis, 2017). Trisomy 21 arises due to a (karyotype) a failure of the segregation of chromosome 21 during either oogenesis or spermatogenesis resulting in a karyotype of 47, XX, + 21 for females and 47, XY, + 21 for males (S. E. Antonarakis, 2017).

In approximately 90–95% of cases, Trisomy 21 originates from meiotic nondisjunction mistake in the maternal meiotic division at the time of oogenesis. As a result, an offspring with DS inherit two maternal and one free copies of chromosome 21. As reported by several studies, non-disjunction occurring in first maternal meiotic division (M-I) is more frequent than the second meiotic division (M-II) (Vranekovic et al., 2012).

The most common cause of chromosomal nondisjunction is advanced maternal age and inaccuracy during recombination. However, other maternal medical conditions such as substance abuse, infection, medications, radiation, hyperthermia, chemical exposure, and uterine abnormalities have also been reported to be associated with meiotic non-disjunction as well as DS (Brent, 1999).

Recent researches are coming up with the evidence of strong association of several lifestyles and environmental factors with the occurrence of meiotic nondisjunction and consequently DS. The lifestyle factors include the maternal smoking, consumption of alcohol, substance abuse, occupation etc and the environmental factors includes exposure to hazardous pollutants, chemical toxicant, pesticides etc.

1.4 Congenital heart defect (CHD)

The defect in cardiac architecture and functioning shares a large burden of birth defect worldwide. Congenital anomalies of the heart include Ventricular Septal Defect (VSD), Pulmonary Stenosis (PS), right ventricular hypertrophy and an overruling aorta. These four conditions are together known as the Tetralogy of Fallot (TOF) that results in a significant morbidity and mortality in newborns (Simeone et al., 2014).
The underlying mechanism of CHD is known to be mutations in regulator genes of cardiac development during embryogenesis. However, epidemiological data suggest that influence of environmental factors also plays a pivotal role in the pathogenesis of CHD. For instance, studies have shown that a prenatal maternal exposure to drugs like ACE inhibitors increases the risk of several congenital malformations and cardiac disease in offspring (D. K. Li et al., 2011). In addition to this, many other factors such as nontherapeutic and therapeutic drug exposure, dietary deficiency, cigarette smoking, febrile illnesses in pregnancy, prenatal alcohol consumption, racial/ethnic variations, reproductive history, paternal/maternal age, infections such as rubella and coxsackie virus are known to be linked with the progression of CHD (Roeters van Lennep et al., 2002). Mutations in various genes namely \( NKX2-5, SMAD3, NTRK3, GATA6, TBX2, TBX18, ATA6, \) and \( TBX2 \) are proven to play a crucial role in developing CHD (A Richards & Garg, 2010; Edwards & Gelb, 2016). In addition, unique copy number variations, and chromosomal aneuploidy such as trisomy 13, 18, or 21 are also known to be some of the major etiologies of CHD. A close association between Trisomy 21 and CHD has been well documented and thus more studies are warranted to delineate the underlying mechanism of this association (Bruneau, 2008).

1.5 Aetiologies and risk factors for birth defects

The major contributing factor that leads to the congenital anomalies is genetic. However, the environment also plays a significant role in developing birth defects in infants. The etiologies can be divided into three main categories as follows:

a) Genetic causes: 25% of congenital abnormalities are estimated to be of genetic origin. This group includes chromosomal abnormalities such as DS and Mendelian single-gene defects (e.g. achondroplasia or Holt-Oram syndrome). Two circumstances that may lead to a higher incidence of congenital abnormalities with genetic origins include the following (Czeizel, 2005):

- Women giving birth after 35 years of age and
- Increased rate of consanguineous marriages

b) Environmental: This includes teratogenic drugs, alcohol, smoking and several environmental pollutants. The percentage of environmental cause may be approximately 15% of total congenital abnormalities (Czeizel, 2005).
c) **Complex (multifactorial) origin:** This group of anomalies is caused by gene-environmental interaction. This type of anomalies results whenever the genetic predisposition (polygenic liability) is elicited by environmental 'risk' factors. The most common examples of congenital abnormalities, such as isolated neural-tube defects, orofacial clefts, cardiovascular malformations, congenital pyloric stenosis, congenital dislocation of the hip, etc. Are included to this group. Approximately 60% of the total congenital abnormalities including the abnormalities with unknown origin belongs to this group of disorder (Czeizel, 2005).

### 1.5.1 Genetic causes

Genetic factors act as a major contributing factor to congenital malformations. The major genetic alterations include chromosome abnormalities, including duplications and deletions, point mutations etc. 15%-24% of the individual with congenital anomalies contains either deletions or duplications of the chromosomes. Point mutations in the genes also have been described in patients with birth defects (Zweier et al., 2007).

#### 1.5.1.1 Genetic rearrangements

Chromosomal imbalances often lead to the birth defects. Numerous genomic disorders are coupled with the congenital heart defects. Particularly, microdeletions at 22q11 and microduplications at 1q21.1 are most widespread congenital malformations which cause heart defects (Sun et al., 2015).

#### 1.5.1.2 Methylation alterations

Methylation alterations play critical role in several diseases including birth defects. DNA methylation and histone modifications both together cause changes in the regulation of gene expression without modifying the nucleotide sequence (Handy et al., 2011). The irregular pattern of DNA methylation results in changes in the transcription process, in turn, affecting the gene expression. Studies have shown the role of aberrant methylation in congenital anomalies such as CHD and DS are associated with unsupervised regulation of epigenetic mechanisms (Magenis et al., 1990).
1.5.1.3 Point mutations

Many single gene disorders leading to congenital malformations are caused by the Point mutations. A mutation in the genes such as GATA4 (Garg et al., 2003) or NKX2-5 (Watanabe et al., 2002), (genes which play an important role in heart development) may cause congenital heart malformation. Point mutation plays a crucial role in not only in CHD but also in other types of malformations, such as holoprosencephal.

1.5.2 Environmental causes

1.5.2.1 Alcohol consumption

Consumption of alcohol during pregnancy may leads to birth defects. It is estimated that 5% to 10% of pregnancies worldwide are currently at risk of alcohol-related birth defects. Intake of excessive amount of alcohol affects the organ systems and causes severe developmental delay due to the Fetal Alcohol syndrome (FAS). Alcohol also contributes to preterm delivery, fetal death and stillbirth (Kancherla et al., 2014).

The second half of the first trimester is the period during which fetal development is most susceptible to the impact of alcohol. Heavy alcohol use during this time is associated with facial findings including an increased risk of smooth philtrum, thin vermillion, microcephaly, and weight and height deficiencies (O'Leary et al., 2010). Overall, there is a greater than 4-fold increase in birth defects occurrence with extensive exposure to alcohol during the first trimester (Jones et al., 2013).

1.5.2.2 Smoking

Smoking during pregnancy is associated with numerous adverse maternal, fetal, and neonatal outcomes. Cigarette smoking is arguably the most important modifiable risk factor during pregnancy. According to the 2014 US birth certificate data, 10.9% of women reported smoking during the 3 months prior to pregnancy. Of those women, 75% continued smoking during pregnancy (Dahlin et al., 2016). The effects of cigarette smoking are multifactorial and are likely confounded by duration/amount, maternal age, genetic susceptibility, and gestational age at exposure.
1.5.2.3 Obesity

For the obese patient, pregnancy can be associated with a wide range of perinatal risks and/or complications. Minimizing these risks and managing potential complications are becoming increasingly challenging for women’s health providers (Harris et al., 2017).

1.5.2.4 Advanced maternal age

Weakened chromosomal cohesion in aging oocytes results in chromosomal segregation errors, serving as the presumed mechanism of aneuploidy. These chromosomal abnormalities result in increased likelihood of infertility, miscarriages, and birth defects. At age 40 years and above, a chromosomal abnormality is found in every 50 births (Duncan et al., 2012). The most common chromosomal abnormalities associated with increased maternal age are trisomies 21, 18, and 13 and sex chromosome abnormalities (Hollier et al., 2000). Down syndrome occurs in 1 in 400 women at age 35 years, 1 in 105 at age 40 years, and 1 in 12 at age 45 years. In addition, increasing maternal age is also associated with a rise in nonchromosomal malformations (Bunt & Bunt, 2014).

1.5.2.5 Advanced paternal age

Advanced paternal age is associated with an increase in sporadic gene mutations for autosomal dominant conditions including achondroplasia and Apert, Waardenburg, Crouzon, Pfeiffer, and Marfan syndromes. These mutations can potentially result in associated congenital anomalies (Harris et al., 2017).

1.5.2.6 Folic acid deficiencies

Folate is a necessary coenzyme in DNA synthesis. During fetal development, cells are undergoing widespread and constant division to sustain the evolution of the growing embryo. In this stage of embryogenesis, folic acid deficiencies may cause various fetal developmental and functional defects (Shaw et al., 1995). According to the previous studies women a daily multivitamin containing folic acid supplements prior to conception and throughout pregnancy can decrease the risk of having children with an orofacial cleft approximately by 25% to 50% (Shaw et al., 1995).
1.5.2.7 Radiation exposure

Multiple exposures to ionizing radiation during pregnancy can cause a varied set of birth defects. The adverse fetal effects of ionizing radiation exposure are to various parameters, such as the gestational age of the fetus at the time of exposure, the dose absorbed by the fetus, and fetal cellular repair mechanisms (De Santis et al., 2007). During organogenesis (approximately 2–8 weeks after fertilization), the developing embryo can be subject to multiple sequelae of ionizing radiation including cell death, abnormalities of cell migration and proliferation, or mitotic delay (De Santis et al., 2007).

1.5.2.8 Drugs

There are a number of medications that should be avoided in pregnancy or that are absolutely contraindicated. Examples of drugs that should be avoided in pregnancy include ACE inhibitors, anticonvulsants, folic acid antagonist, statins etc.

Studies have shown that first-trimester exposures to ACE inhibitors are related to the development of major congenital malformations. Exposed infants had a high risk of cardiovascular, renal, and central nervous systems defects. Cardiac defects included atrial septal defect, patent ductus arteriosus, ventricular septal defect, and pulmonic stenosis (Cooper et al., 2006). Importance of folic acid supplements has already been discussed in several studies. In addition, taking folic acid antagonist increases the risk of neural tube defect, oral defects and oral cleft (Hernandez-Diaz et al., 2000).

1.5.2.9 Socioeconomic and demographic factors

Other than the risk factors discussed, socioeconomic status of the parents also plays a significant role in the development of the congenital anomalies. According to (Hunter et al., 2013) low maternal socioeconomic status is strongly associated with meiosis II chromosome 21 nondisjunction. Studies have shown that approximately 94% of the congenital anomalies occur in under developed countries. Poor access to adequate healthy food, exposure to infectious agents and alcohol consumption and inadequate health care delivery arrangement are some of the factors that aggravate the situation (“WHO | Congenital Anomalies”, 2017).
1.5.2.10 Infections

Infections during pregnancy can also enhance the threat of birth defects. Some of the examples of the infections that may lead to the congenital malformations in infants are as follows (Zheng et al., 2004):

a) Rubella infection or German measles a viral infection: this infection during pregnancy can result in miscarriage, deafness, intellectual disability, heart defects and blindness in infant (White et al., 2012).

b) Loss of hearing, vision trouble and intellectual disability are associated with mother with toxoplasmosis infection during pregnancy.

c) Sexually transmitted infections such as syphilis and cytomegalovirus may results in serious birth defects. For example, certain birth defects such as microcephaly and other abnormalities may occur if, mother had Zika virus infection during pregnancy (Rather et al., 2017).

1.6 Rationale of the study

There are significant numbers of DS patients in south Gujarat region where this study was carried out. However, the incidence needs to be correlated in terms of social factor like environmental factors viz., lifestyle or occupational exposure to known hazardous compounds in order to establish the customized pattern of genetic counselling and risk assessment for the region. In addition, data obtained in this study should be compared with the previously published reports across the world.

From an array of previous studies, it is known that a range of environmental exposures influences the epigenetic status, including diet and nutrition and lifestyle factors. Out of these known maternal risk factors for CHD, many are able to trigger changes in the epigenetic state during parental development.

The epigenetic influences in CHD are still poorly studied and have the prospective to make unique contributions to our understanding of the pathogenesis of this important group of anomalies. Therefore, the epigenetic impact on the inheritance of such birth defects should be
studied in depth to diagnose, modify and manage the morbidities associated with the conditions. In addition, currently, there are no proven biomarkers available in clinical practice for the pre-or post-natal detection of CHDs. Given the clinical significance of CHD and the frequency of missed or late diagnosis, this is a major deficiency.

In previous studies, it was observed that cytosine methylation status can act as a possible biomarker for the diagnosis of multiple categories of CHD (Radhakrishna et al., 2016). Our objective, therefore, in this proof of concept study was to evaluate the efficacy of cytosine methylation in placental DNA of the detection of CHD. Further, we aimed at using cytosine methylation to investigate the molecular pathogenesis of non-syndromic VSD based on the associated genes and gene pathways that were differentially methylated in the VSD cases. Our additional objective was to analyze another important epigenetic mechanism related to microRNA (miRNA) that exerts control over DNA methylation and suppresses the expression of other genes. Recent data suggest an important role of miRNA in CHD development (T. Smith et al., 2015). We therefore also evaluated methylation status of known microRNA genes. Like many other genes, we reasoned that DNA methylation status of miRNA gene should correlate with miRNA expression levels. To our knowledge, there are no prior reports used placental molecular analysis for the detection of investigation of CHD pathogenesis. In that respect, this is a milestone study to evaluate the same.

A.7 Aims and Objectives

A)

1) To study the occurrence of trisomy 21 by constitutional karyotyping in DS subjects.
2) To study the parental and meiotic origin of DS using STR Marker by QF-PCR (Quantitative Fluorescent Polymerase Chain Reaction).

B)

1) To determine the genome-wide DNA methylation profiles associated with CHD in nonsyndromic cases, Ventricular Septal Defects (VSD) and Tetralogy of Fallot (TOF).
2) Validation of candidate pathogenic mutations and their genetic susceptibility factors to CHD in nonsyndromic cases.
3) Integrate genetic and network analysis to underlying etiology of the CHD phenotype.
1.8 Summary of the thesis

Epidemiological studies have shown that congenital anomalies and their associated morbidities are a major concern worldwide. Besides the clinical difficulties, birth defects in offspring exert a psychosocial impact in both the child and its parent.

In this context, this study aimed at redefining the etiology and risk factors for highly prevalent birth defects such as DS and CHD. For DS, it was found that in contrary to existing idea, environmental and lifestyle factors might have played a vital role in paternal age in developing DS. The risk of occurrence of DS was independent of parental age, however, the present occupation and environmental exposure might have played a pivotal role in risk calculation. Constitutional karyotyping in 120 subjects confirmed the occurrence of DS in all the patients where most of them are from young parents. The data clearly showed occupation like diamond polishing and agriculture may have potential risk of incidence of DS due to parental exposure to hazardous chemicals such as pesticides used in agriculture. Moreover, an analysis of STR markers by QF-PCR determined the aneuploidy accurately by relative allele dose quantitation. The result showed the 95% of aneuploidy is of maternal origin and out of which 92% of the non-disjunction occurs due to the maternal meiotic error I (MMI). Again the advanced parental age was statistically insignificant as a risk factor for developing Trisomy 21.

Similar to DS, CHD is associated with a considerable morbidity and mortality. This study aimed at delineating the epigenetic variations and the genes involved in novel signaling pathways in the pathogenesis of congenital heart defects. Specifically, we focussed on the determination of genome-wide DNA methylation profiles associated with CHD, validation of candidate pathogenic mutations and their genetic susceptibility factors to CHD and integrating the genetic and network analysis to underlying etiology of the CHD phenotype.

The result of genome-wide methylation profile analysis revealed 69 novel cytosine loci whose degree of methylation is possibly related to the incidence of TOF. In summary, the study was able to identify 8 novel highly significant genes for TOF, 39 highly differentially methylated CpG loci and moreover, an analysis of the GDAC FIREHOSE database indicated a strong correlation of expression of genes engaged in the pathogenesis of TOF with the differential
methylation status. Therefore, this study provided insights into the pathogenesis of TOF and generated a novel and precise putative biomarkers for TOF detection and established the role of epigenetic modification in transgenerational inheritance of some congenital defects such as DS and CHD.

Bearing in mind the significant role of epigenetics in the regulation of gene expression and the rising in numbers of evidences connecting epigenetic alterations with congenital malformations, we have looked into potential abnormal methylation profiles in DNA isolated from placenta of samples with VSD and compared it with controls. Our data results showed a role of methylation status in VSD subjects. We found changes in levels of methylation in 8 miRNAs independently or in combination affecting 160 genes identified in the present analysis. Gene ontology analysis identified several key biological processes and functions for these differentially methylated genes that are known to be associated with heart development or heart diseases.