ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF ANATOMY

PhD Dissertation

Investigations of Visible Congenital Anomalies at Birth and Associated Factors in Southwestern Ethiopia

By: Soressa Abebe Geneti

July 2021

Addis Ababa, Ethiopia

Investigations of Visible Congenital Anomalies at Birth and Associated Factors in Southwestern Ethiopia

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A dissertation submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the award degree of Doctor of Philosophy (Ph.D.) in Medical Anatomy

Declaration

I hereby declare that this dissertation work is my original work and has never been presented for an award for degree from this University and any other University

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Acknowledgements

I am very much grateful to the persistent guide, encouragement and support of my principal advisor Dr. Girmai Gebru, without whom this dissertation would not have been successfully accomplished. I extend my gratitude for his unreserved, constructive and invaluable comments from the very first day of the research project till its completion. I am very much grateful to my co-advisors Dr. Demisew Amenu and Professor Zeleke Mekonnen for their keen interest, unreserved guidance and consistent efforts for the successful completion of this dissertation. I am highly indebted to Mr. Lemessa Dube who helped me in organizing the project, data entry and statistical analysis. My special thanks go to Dr. Girma Seyoum for his invaluable encouragement and support during my course of study till completion. My deepest gratitude also goes to Mr. Bacha Tilahun, Mr. Berecha Dugasa, Mr. Ashanafi Gebregzabeher, Mr. Birehanu Namomsa and all staff members of the study hospitals for their help during the study.

I would like to thank all medical wards of each study hospital in southwestern Ethiopia who helped me in providing information necessary for the study. I would like to extend my sincere thanks to the Jimma University Specialized hospital, Shanene Gibe hospital, Limu Genet hospital, Metu Karl hospital, and Nekemte hospital, which have allowed and helped me during data collection. My gratitude goes to my trained data collectors at each hospitals of the study area. My thanks also is extended to the staff of the department of Gynecology and Obstetrics of each hospital who had direct or indirect inputs for the successful accomplishment of the study. I extend my gratitude to Dr. Zewdu Terfework for his unreserved guidance and help during laboratory work and DNA analysis to detect genetics of birth defects. My thanks also goes to Tigist Tsegaye and Tizibit Yefrashewa for their assistance during laboratory processes.

My heartfelt gratitude should also go to all academic and technical staff of the Department of Anatomy for their unreserved assistance in one way or another. I greatly acknowledge the School of Graduate Studies, Addis Ababa University, CDT – Africa and Jimma University for funding the research project.

Last but not least my special honor and thanks is extended to my wife Gete Oljira and my daughters Mersimoi Soressa, Meti Soressa, Simbo Soressa and my son Beki Soressa for their continuous and consistent encouragement.

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List of abbreviations

ANC	Antenatal Care
AOR	Adjusted Odds ratios
CA	Congenital Anomaly
CDH	Congenital Diaphragmatic Hernia
CHD	Cardiac Heart Defect
CI	Confidence Interval
COR	Crud Odds Ratios
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FISH	Fluorescence *In Situ* Hybridization
GP	General Practitioner
ICD	International Classification of Diseases
JMC	Jimma Medical Centre
MLPA	Multiplex ligation-dependent probe amplification
NTD	Neural Tube Defect
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
USA	United State of America
USD	United State Dollar
WHO	World Health Organization

List of manuscripts

1. Abebe S, Sori DA, Gebru G, Mekonnen Z, Dube L. **Risk factors associated with congenital anomalies among newborns in southwestern Ethiopia: A case – control study.** PLoS ONE 16(1): e0245915. https://doi.org/10.1371journal.pone.0245915

2. Geneti SA, Gebru G, Sori DA, Mekonnen Z, Dube L. **Prevalence and patterns of birth defects among newborns in southwestern Ethiopia: Cross sectional retrospective study**. **Under review (Pan African Medical Journal)**

3. Geneti SA, Gebru G, Sori DA, Mekonnen Z, Dube L**. Incidence of congenital anomalies and associated maternal factors among newborns in southwestern Ethiopi**a: Descriptive cross sectional study (**Manuscript under preparation**)

4. Geneti SA, Gebru G, Sori DA, Mekonnen Z, Dube L. **Prevalence of neural tube defects and associated factors among newborns in southwestern Ethiopia: Case – control study (Manuscript under preparation)**

5. Geneti SA, Gebru G, Sori DA, Seyoum G, Terfewor Z, Mekonnen Z. **Chromosomal abnormalities among mothers of affected newborns with congenital anomalies in southwestern Ethiopia: Genetic study (Manuscript under preparation)**

Abstract

Background: Prenatal development can be considered as normal or abnormal. Abnormal development occurs because of interference of normal development from genetic disorders, environmental factors or the combination of genetic and environmental factors during the critical period of embryogenesis with which the neonate is born with a disorder that is described as congenital anomalies. Congenital anomaly is a series of structural, functional and metabolic abnormalities present at birth because of several causative agents during critical period of intrauterine life.

Congenital anomalies highly contribute to abnormal intrauterine development that leads to prenatal disturbance and postnatal morphological defects that may persist throughout life. According to World Health Statistics, about 260,000 neonatal deaths worldwide are caused by congenital anomalies. The frequency of congenital anomalies in developing countries including Ethiopia is underestimated because of the deficiencies in diagnostic capabilities and lack of reliability of medical records and health statistics. Instead, the available recorded diagnoses in vital health statistics relay on obvious illnesses, rather than on preexisting congenital anomaly present at birth contributing to infant mortality and morbidity leading to the significant community problem. Therefore, it is vital to evaluate types, prevalence, incidence and predisposing risk factors which may provide a baseline for public health plan, suggesting protective strategies against congenital anomalies and establish genetic counseling if the defects are of genetic origin and provide input for further investigation.

Objective: To assess the prevalence, incidence, types, and patterns of congenital anomalies or disorder at birth and associated risk factors among newborns in southwestern Ethiopia

Methods and materials: The study was conducted in southwestern Ethiopia. This research used quantitative study methods and genetic analysis. The quantitative methods included institution based retrospective cross-sectional, descriptive cross-sectional and case-control study. The study was conducted from 2011 to 2015 during which 45,951 deliveries were attended for the retrospective document review study. All records of births that occurred in the selected hospitals during the study period were identified from medical records. Descriptive

cross sectional study was also conducted in those six selected hospitals from 2016 to 2018, during which 35,080 deliveries were attained. A prospective registration of all delivers of any gestational age during the study period was conducted to determine the incidence of congenital anomalies. Newborns either still or life birth was evaluated for the presence of any congenital anomalies. Data was collected using structured and standard checklist. Case-Control study was conducted on newborns and their mothers within the period of 2016 to 2018 in those selected hospitals. Data were collected after evaluation of the neonate for the presence of congenital anomalies using the standard pretested checklist. The data was analyzed using SPSS version 25.0. P <0.05 was set to be statistically significant. Chromosomal abnormities were also detected in their sub telomeres region using multiple ligation probe dependent amplifications method with help of probe mix.

Results

The prevalence of the birth defects at birth was found to be 5.5 per 1000 births. Of a total of 35,080 neonates delivered during the study period, 0.72% of them had congenital anomalies. The overall incidence of congenital anomalies in southwestern Ethiopia was 71.6 per 10,000 births. Risk factors such as unidentified use of medications or drugs in the first three months of pregnancy (AOR = 3.435; 95% CI: 2.286 – 5.160), exposure to pesticide (AOR = 3.926; 95% CI: 1.659 – 9.289), passive smoking (AOR = 4.104; 95% CI: 2.277 – 7.397), surface water as sources of drinking (AOR = 2.073; 95% CI: 1.385 – 3.100) were significantly associated with the occurrence of congenital anomalies. Similarly, risk factors such as passive smoking (AOR = 4.171; 95% CI: 2.272 – 7.681), exposure to pesticide (AOR = 3.823; 95% CI: 1.606 – 9.106), and maternal diabetes mellitus (AOR = 16.381; 95% CI: 1.614 – 166.241) were significantly associated with the occurrence of neural tube defects. Multiple ligation dependent probe amplification analysis confirmed the presence of deletion in the chromosome bands of 3q29, 8q24.3, 9q34.4, 15q11.2, 15q26.3, 18p11.32, 19q13.43, Xq28 and duplications of 3p26.3 and 6p25.3 in sample population tested for chromosomal abnormalities

Conclusions

Nearly equal proportions of birth defects occurred among male and female newborns. The most frequent congenital anomaly was neural tube defects followed by musculoskeletal defects. From frequency distribution of associated factors, mothers who had neonates with congenital anomalies were exposed to passive smoking, pesticides, unidentified usage of medicines during the first three months. Poor folic acid supplementation was more frequent among the neonates delivered with congenital anomalies. Risk factors such as passive smoking, exposure to pesticides, chemicals and use of surface water as a source of drinking during early pregnancy had a significant association with the occurrences of congenital anomalies. As the congenital anomalies were found to be the main causes of infant mortality and morbidity, there is a need for an urgent intervention to control the cases and there is a need to continuously provide health information for the community on how to prevent and control predisposing risk factors. Chromosomal abnormalities were evident in sub-telemeric regions of some sample population.

Chapter One

1. Introduction

1.1 Background of the study

Human development begins when a spermatozoon fuses with an ovum to form a single totipotent cell known as a zygote. A series of cell divisions transform the zygote into blastocyte which eventually implants into a functional layer of the maternal endometrium at the end of the first week or the beginning of the second week of the zygote formation. Embryonic development involves differentiation, cell division, rearrangement, and programmed cell death-apoptosis to form cells, tissues, organs, and organ systems and can be decribed in three periods (Moore et al., 2011).

Among these, embryonic period (3^{rd} – 8^{th} weeks) is considered to be critical and vulnerable period of development as any abnormal interference with differentiations of the primordium of organ systems lead to abnormal development. From ninth to fourteenth weeks is the period where differentiated structures are highly specialized and grow in size and is a less vulnerable period of intrauterine life as differentiation of most of the organ system is over during this phase (Sadler, 2012; Moore et al., 2011).

Prenatal development can be considered as normal or abnormal. Abnormal development occurs because of interference of normal development from genetic disorders, environmental factors and the combination of both genetic and environmental factors (multifactorial) during the critical period of embryogenesis that leads to abnormal cytogenesis, histogenesis, and morphogenesis with which the neonate born with disorders is described as congenital anomalies (CAs) present at birth (Butt et al., 2013; Rizk et al., 2014; Victor, 2002). CAs is structural as well as functional abnormality present at birth due to several causative agents that interfere with the developmental processes of embryogenesis during the critical period of intrauterine life (Zhang et al., 2012).

However, little is known about the causes of CAs (Kucik et al., 2012) literature suggested that 20% may be due to a combination of heredity and environment factors; 7.5% may be due to

single-gene mutations; 6% is attributed to chromosome abnormalities, and 5% may be due to maternal illnesses, such as diabetes, infections or anticonvulsant drugs (Rizk et al., 2014). Approximately 40% to 60% of CAs are of unknown origin (Ahmadzadeh et al., 2008; Victor, 2002).

CAs are structural abnormalities present at birth that can be described in terms of four clinically significant types based on the cause, timing, and extent of the developmental interferences during prenatal life by teratologic agents. These are namely: Malformation, disruption, deformation, and dysplasia (Ekwere et al., 2011; Ahmadzadeh et al., 2008).

Malformation is morphologic defect of an organ, part of an organ, or larger region of the body which results from an inherently abnormal developmental process (Eluwa et al., 2013; Ekwochi et al., 2018). This indicates that the developmental potential of the primordium is abnormal from the beginning, such as in the case of chromosomal abnormality of a gamete at the time of fertilization (Rizk et al., 2014). CA is an exciting problem for study because of the high incidence of their occurrences and the upsetting effect on the individual and family as well as the community (Eluwa et al., 2013).

1.2 Statement of the problem

CAs are considered to be the major health problems, and the quality of their treatment is an indicator of the quality of public health concern (Eluwa et al., 2013). Eluwa et al. (2013) indicated that CA highly contributed to abnormal embryogenesis that leads to prenatal disturbance and postnatal morphological defects that may persist throughout ones life. Moreover, abnormal morphogenesis leads to CAs that have many recognized causes, including specific single-gene mutations, chromosome imbalances, and the action of teratogenic agents (Rizk et al., 2014; Detrait et al., 2005).

The frequency of CAs in developing countries including Ethiopia is underestimated because of the deficiencies in diagnostic capabilities and lack of reliability of medical records and health statistics. Instead, the available recorded diagnoses in vital health statistics relay on obvious illnesses, rather than on preexisting congenital defects present at birth that increase infant mortality and morbidity leading to the significant community problem (Ekwere et al., 2011; Waqas et al., 2009).

Existing limited information about CAs in Ethiopia shows that factors predisposing to congenital disorders are commonly high. However, available epidemiological data on CAs, genetic diseases/disorders in Ethiopia is limited. Few health facilities - based studies conducted indicated that some gross malformations such as cleft lip and palate, congenital heart diseases, club-foot, and other systemic malformations such as neural tube defects and gastro-intestinal malformations are identified (Bekele et al., 2019).

In Ethiopia, chemicals such as pesticides and fertilizers are highly utilized. This practice may add chemicals to the soil and surface water which may consequently increase the chance of abnormal intrauterine development. Among those populations living near waste disposal sites in an industrial area and contaminated land, pesticide exposure in agricultural areas, air pollution and industrial pollution sources, food contamination, and disasters involving accidental, negligent or deliberate chemicals released in its great amount is significantly increasing which may contribute to abnormal embryogenesis (Kurdi et al., 2019; Calzolari et al., 2014; Hussain et al., 2014; Parmar et al., 2010). Moreover, the genetic factors have their great share in abnormal intrauterine development leading to several malformations (Boyd et al., 2011).

Investigations of the incidence, types, forms, and severities of different malformations and genetic disorders as well as the causative agents that are considered to be risk factors need to be evaluated. Therefore, the present study aims to investigate the incidence, types, and forms of CAs or anomalies as well as the associated risk factors in southwestern Ethiopia.

1.3 The rationale of the study

CAs largely contribute to infant mortality and morbidity and are the major causes of infant life lost each year because of inadequate medical care and underestimation of the defects. Earlier recognition and reactions to CAs often may provide protective majors for severe health problems.

Regardless of the health burden of CAs, very few studies were conducted in Ethiopia. Only a limited number of such studies were conducted in the country with more than 100 million population size. No study was conducted in southwestern Ethiopia, Oromia regional state. This indicates that infant mortality and morbidity due to CAs is underestimated.

There is no clear number of infants born with CAs in health institutions. Furthermore, there is data scarcity on CAs in Ethiopia due to limited surveillance system and registry at national, regional, zonal, and district levels revealing the absence of updated database. Moreover, understanding the etiology of CAs is very essential for health care plan, clinical and research purposes.

Therefore, it is vital to evaluate the types, prevalence, and incidence of CAs and their predisposing risk factors which may provide a baseline for public health plan and protective strategies against CAs and establishment of genetic counseling and provide provision of input for further investigation.

1.4 Significance of the study

CAs are the fifth leading causes of years of potential life lost and they are the major causes of morbidity and mortality throughout the world. Regardless of their clinical importance, there are very few studies conducted directly related to predisposing risk factors including genetic diseases/disorders that could demonstrate the incidence and prevalence level of these conditions and related risk factors. Hence, to realize the organization of community genetic services program at the primary health care level in Ethiopia, conducting community-based studies may give a clear picture of predisposing factors and prevalence of congenital disorders. Quantification of CAs within a given population is of paramount importance for estimating and documenting the need for prevention, for public health policy development, for planning and

implementation of services needed by children with malformations and for evaluating the effects of preventive measures and treatment services.

So far, there is no research published regarding the incidence of CAs and its associated risk factors in southwestern Ethiopia. The present study was planned to investigate the prevalances, incidences, types, forms, and patterns of CAs among stillbirth and live birth infants and predisposing risk factors in southwestern Ethiopia

Chapter Two

2. Literature review

Intrauterine development can be considered as normal development as well as abnormal development. Abnormal development occurs because of the meddling of normal development from genetic disorders, environmental factors, and the combination of both genetic and environmental factors during the critical period of embryogenesis. These factors may lead to abnormal cytogenesis, histogenesis, and morphogenesis with which a neonate may be born with a defect/defects that are described as a CAs (Butt et al., 2013; Victor, 2002).

2.1 Prevalence of congenital anomalies

Major CAs are common, critical and costly. Collectively, they happen in one in 33 births, which accounts to an estimated rate of 7.9 million babies worldwide (Feldkamp et al., 2017; Christianson et al., 2006). The prevalence of CA varies among different ethnic groups (Delport et al., 1995). Extensive literature is available on the incidence of CAs in first-world countries and studies have also been done in several third-world countries (Delport et al., 1995). However, in Africa south of the Sahara, limited information is available on the incidence of CAs. Previous studies had limitations in that they were either retrospective, performed on small sample numbers, or they reported the frequency of a single abnormality or a few specific abnormalities (Butt et al., 2013).

CAs are emerging as important prenatal problems contributing to prenatal mortality and morbidity (Abdou et al., 2019; Parmar et. al., 2010). The difference between the frequency of types of CA in different parts of one country and reports from other countries may be due to genetic background and geographic nutritional and socioeconomic differences. More investigations are needed to determine the factors underlying the various types of CAs encountered in a given area (Samadirad et al., 2012; Parmar et. al., 2010).

CAs also have implications in society as they lead to an increase in the incidence of stillbirth and neonatal death. Inherited and chromosomal anomalies are associated with loss of physical or mental and intellectual abilities as well (Rizk et al., 2014; Samadirad et al., 2012; Parmar et. al., 2010).

CAs begins to emerge as one of the major childhood health problems as well as the cause of infant mortality and morbidity throughout the world especially in developing countries (Almeida et al., 2016; Rizk et al., 2014). In most cases, infants with malformations do not survive, more than 70% die in the first month of life. Moreover, treatment and healing of children with CAs is costly and complete recovery may be impossible (Mohamed et al., 2013; Taboo, 2012; Amany et al., 2011; Naeimeh et al., 2010; Vinceti et al., 2006).

According to World Health Statistics, about 260,000 neonatal deaths worldwide are caused by CAs (Rizk et al., 2014). This figure represents about 7% of all neonatal deaths but ranges from 5% in the South-East Asia region to more than 25% in the European region. It was also indicated that between-country variation: from 4% (Bangladesh, Equatorial Guinea, Ethiopia, Liberia, Mali and Sierra Leone) and an estimated 8% in China to 38% and more in (Bahrain, Cyprus, Ireland, Kuwait, Qatar and the Syrian Arab Republic) (Rizk et al., 2014).

Based on a 2010 global burden of disease study, CAs accounted for 510,400 deaths worldwide with 1% of all deaths and attributed to becoming the 23rd cause of all deaths (Lozano et al., 2012; Murray et al. , 2012). On the other hand, death due to CA is likely to be varied in early life, the burden in year lost is higher and is ranked as 14th among the causes of all deaths worldwide (Lozano et al., 2012). According to Boyle et al.(2018), 17% to 43% of neonatal death was accredited to CAs with higher rates record in Malta (43%) and Ireland (42%).

According to eleven EUROCAT countries (19 registries), recorded rates of infant mortality because of CAs was 80% (Boyle et al., 2018). Furthermore, the average prevalence of CAs cases of termination of pregnancies in these eleven countries was 26.9 per 1000 births. Besides, a total of 17% of terminations of pregnancies were because of CAs. The prevalence CAs was 4.6 per 1000 births, varying from 0 in Malta to 7.5 per 1000 in Spain-Basque Country. A total of 2.1% were stillbirths, with a prevalence of 0.6 per 1000 births although varying among countries (Boyle et al.,2018). The same authors demonstrated that early neonatal deaths accounted for 49% of all first-year deaths. Neonatal mortality was highest for multiple anomalies live births, 15% of cases, and lowest for cases with a single anomaly nearly with only 4% (Christianson et al., 2006)

CAs are worldwide problems, although their impact is particularly more severe in low and middle-income countries where more than 94% of the births with severe defects and 95% of the deaths of these infants occur (Christianson et al., 2006). Nearly about 7.9 million neonates are born with major CAs each year (Ndibazza et al., 2011). However, the proportion of global neonatal mortality due to these defects increased from 3% in 2008 to 4.4% in 2013 (Liu et al., 2015; Oestergaard et al., 2011).

Major CAs are considered to be anomalies with a significant effect on the life expectancy of the newborns and occurred in 2–3% of live births and 20–30% of stillbirths (Kumar, 2008). According to WHO, nearly 3.2 million of birth defect-related disabilities and an estimated 276,000 infant deaths occur each year. The overall prevalence of BD was 29.2 per 1000 live births of which 51% were Caucasians (Egbe et al., 2015).

There are reports which indicate a great variations among different ethinc groups of the world. The risk of overall CA was lower in African – American as compared with Caucasians and Hispanics. In general, the prevalence of CA was similar in Caucasians and Asians. African Americans had a lower risk of cardiac, genitourinary, and craniofacial malformations relative to the Caucasians. However, a higher prevalence of musculoskeletal anomalies among Africans (Wokpeogu et al., 2018; Palma et al., 2013). Hispanics had a lower prevalence of gastrointestinal and genitourinary defects (Wokpeogu et al., 2018).

Asians had a higher risk of craniofacial and musculoskeletal defects (Egbe et al 2015). Concerning specific risks, (the ventricular septal defect, lower urinary tract obstruction, hypospadias, neural tube defect (NTD), cleft lip, and cleft lip-palate) were less prevalent in African-Americans whereas the congenital hip dislocation and congenital foot anomalies were more prevalent in African- Americans compared with Caucasians (Yilmaz et al., 2019; Egbe et al 2015; Flores et al., 2014).

Hypospadias and upper gastrointestinal defects were less prevalent in Hispanics while atrial septal defect, renal dysplasia, and omphalocele/gastroschisis were more prevalent in Hispanics. Asians experienced a higher risk of lower urinary tract anomaly, and congenital hip dislocation, although a lower risk of ventricular septal defect and hypospadias were comparable with Caucasians (Yu et al., 2019). According to a systematic review conducted by Adane et

al.(2020), the overall prevalence of CAs among 25 eligible countries for the study in sub-Saharan was found to be 20.40 per 1000 births.

The highest prevalence was 85.41 per 1000 in Nigeria (Nnadi and Singh, 2017) whereas the lowest was recorded in Gabon with a frequency of 1.43 per 1000 births (Amany et al., 2011). According to a meta-analysis from the studies done in Sub-Saharan African countries, the prevalence of CAs was 43 per 1000 births in southern African region followed by central African with the prevalence of 30.74 per 1000, Eastern Africa 17.30 per 1000 and Western Africa with 9.17 per 1000 (Adane et al, 2020).

Besides, Adane et al. (2020) indicated that musculoskeletal defects were the most frequent types of CAs with the prevalence of 3.90 per 1000 followed by NTDs 2.98 per 1000, cardiovascular system defects 2.83 per 1000, gastrointestinal defects 1.50 per 1000, oro-facial clefts 1.27 per 1000, urogenital system defects 0.69 per 1000 and Down syndrome 0.62 per 1000.

The magnitude of CAs is alarmingly increasing in central and northwest Ethiopia, where orofacial cleft and NTDs contributed for two-third of the total defects (Taye et al., 2016). Taye et al. (2019) also indicated that the overall prevalence of CAs in central and northwestern Ethiopia was 1.99% and described the types of CAs as NTDs, orofacial clefts, masculo-skeletal system anomalies, syndrome disorders, and cardiovascular system defects in order of their prevalence. Another cross-sectional study conducted in three referral hospitals in Addis Ababa showed that the prevalence of NTDs was 63.4 per 10,000 births (Gedefaw et al., 2018).

The evidence of CAs in southwestern Ethiopia is scarce. This study tried to fill the gap by investigating CAs in southwestern Ethiopia, Oromia regional state.

2.2 Associated factors (Causes of birth defcts)

Understanding the etiology of CAs of epigenetics and genetic origin is of paramount importance to control the CAs cases and contribute to the reduction of infant mortality and morbidity, thereby reducing the health burden of a community. Most of the risk factors of CAs are uncertain; however, genetic factors, environmental factors, and multifactorial inheritance are the predisposing risk factors that lead to abnormal prenatal development (Glinianaia et al., 2017; Mohamed et al., 2013; Lobo & Zhaurova, 2008).

2.2.1 Genetic causes of congenital anomalies

Six percent of worldwide births are born with very serious BDs each year with an estimated 7.9 million infants. Though some CAs are controllable and treatable, an estimated 3.2 million of the children are disabled for life. Although some BDs are inherited, others are a product of harmful environmental factors known as teratogens, and still, others are multifactorial, resulting from a complex interactions between genetic and environmental influences (Christianson et al., 2006).

Genetic causes are described in three general categories: chromosomal abnormalities, single-gene defects, and multifactorial influences where the prenatal environment can play a major role in the development of defects, especially those linked to multifactorial causes (Lobo & Zhaurova, 2008). The genetic component of the developing embryo is determined at the time of fertilization. Similarly, it is during this conception that the genetic causes of several BDs are determined (Lobo & Zhaurova, 2008). Chromosomal abnormalities can be defined as structural and numerical abnormalities of the chromosomes that may cause genetic disorders (Victor, 2002).

Structural abnormalities are when part of an individual chromosome is missing, extra switched to another chromosome or turned upside down which may include: **deletion**-A portion of the chromosome is missing or deleted, **duplication**-A portion of the chromosome is duplicated resulting in extra genetic material, **inversion**-A portion of the chromosome has broken off, turned upside down, and reattached. As a result, the genetic material is inverted. **Ring formation**-A portion of a chromosome has broken off and formed a circle or ring. This can happen with or without loss of genetic material, and **translocation**- A portion of one chromosome is transferred to another chromosome. There are two main types of translocations. In a reciprocal translocation, segments from two different chromosomes have been exchanged. In a robertsonian translocation, an entire chromosome has attached to another at the centromere (Samadirad et al., 2012).

One of the significances of fertilization is the restoration of diploid chromosomes obtained from the fusion of the haploid chromosome number of both parents. The normal number of chromosomes in developing human embryo is 46. Any deviation from this normal number of

the chromosome is considered to be numerical abnormalities (Samadirad et al., 2012). Numerical chromosomal abnormality is either autosomal or sex chromosomal abnormalities. The most common autosomal abnormalities include trisomy 21 (Down syndrome), trisomy 13 (Patau syndrome), and trisomy 18 (Edwards syndrome). Developing embryos with these three common conditions can develop very serious disabilities in spite of the environmental factors associated with pregnancy. However, infants born with trisomy 13 and trisomy 18 often die immediately after birth, while infants born with trisomy 21, generally have a relatively long life span (Christianson et al., 2006).

Moreover, a single gene mutation also contributes to a genetic related CAs and is considered to be a predisposing risk factor for the development of CAs (Victor, 2002). Maternal chromosomal abnormalities, which can be defined as numerical and structural abnormalities of the chromosomes may cause genetic disorders. Moreover, a single gene mutation also may contribute to a genetic related CAs (Butt et al., 2013; Victor, 2002). In general the prevalence rates of all genetic CAs collectively range from as high as 82 per 1,000 live births in low – income countries to as low as 39.7 per 1,000 live births in high – income regions (Butt et al., 2013, Godwin et al., 2008).

2.2.2 Environmental factors

The human embryo is well protected in the uterus by the extra-embryonic membranes, although teratogens/ environmental factors may cause developmental disruptions after maternal exposure to them in a specific period of embryogenesis during the critical period in early pregnancy (Mohamed et al., 2013). The organs or parts of an embryo are most sensitive to environmental factors during periods of rapid differentiation leading to abnormal development or malformation of those organs (Shaw et al., 2003; Neumann et al., 1994).

Environmental factors that are considered to be potential risk factors in causing CAs include maternal infection, maternal age, and maternal drug intake during the critical period of embryogenesis. Substances such as caffeine, nicotine, commonly used medicines, maternal nutritional and health status, maternal exposure to hazardous wastes, and maternal alcohol intake during early pregnancy largely contribute in development of CAs (Butt et al., 2013; Shaw et al., 2003). Moreover, parental race, parental socioeconomic status, hyperthermia during early

pregnancy, and maternal diabetes, as well as obesity are considered to be associated risk factors in causing developmental malformations (Victor, 2002).

About 10% of CAs are caused due to environmental factors. The risk identification for exposure to such factors is of paramount importance as this form of CAs can be prevented to a large extent if appropriate caution is taken (Butt et al., 2013).

Chemical substances such as mercury, lead and arsenic are known to lead to the development of CAs (Shaw et al., 2003). Mercury, which is found in some types of fish, has been linked with the development of neurological problems resembling cerebral palsy, as well as mental retardation. Lead has been associated with fetal growth restriction and neurological disorders (Victor, 2002).

Other environmental factors such as radiation also contribute to the formation of abnormal development. For example, X-ray can cause problems with fetal development, such as spina bifida, cleft palate, blindness, abnormalities of the arms and legs, or microcephaly. But the type of malformation or abnormality that develops depends on the dose of X-ray that the pregnant woman is exposed during early pregnancy (Mohamed et al., 2013).

2.2.3 Multifactorial inheritances

Multifactorial inheritance means that many factors are involved in causing CAs. The factors are usually both genetic and environmental, where a combination of genes from both parents, being affected by adverse environmental factors, produce the trait or condition where one gender is often affected more frequently than the other (Neumann et al., 1994).

Multifactorial inheritances are responsible for several developmental disorders resulting in CAs and they are linked to the causation of CAs in humans. They include gene-gene and gene-environment interactions (Graham et at., 2005; Neumann et al., 1994).

Errors in developmental mechanisms and genetic interactions of neural tube formation to take place within a critical period during embryogenesis lead to failure of neural tube closure. As a result, the embryo develops an open neural tube at a specific site of neural tube along its longitudinal axis. Therefore, the failure of the neural tube to close at a specific closure site gives rise to a birth defect known as NTD (Blom et al., 2006).

NTDs are CAs that involve the failure of the neural tube closure during the early phases of development at any level of the rostrocaudal axis of the developing embryo (Au et al., 2010; Mitchell, 2005). The failure of the NTD to close may be because of genetic factors or environmental factors or multifactorial inheritance (Frey and Hauser, 2003). Epidemiological studies provide an opportunity to identify risk factors for NTDs, such as dietary or teratogenic agents, to which susceptibility may be modified by genetic predisposition (Carmichael et al., 2009; Boyles et al., 2005; Mitchell, 2005; Frey and Hauser, 2003).

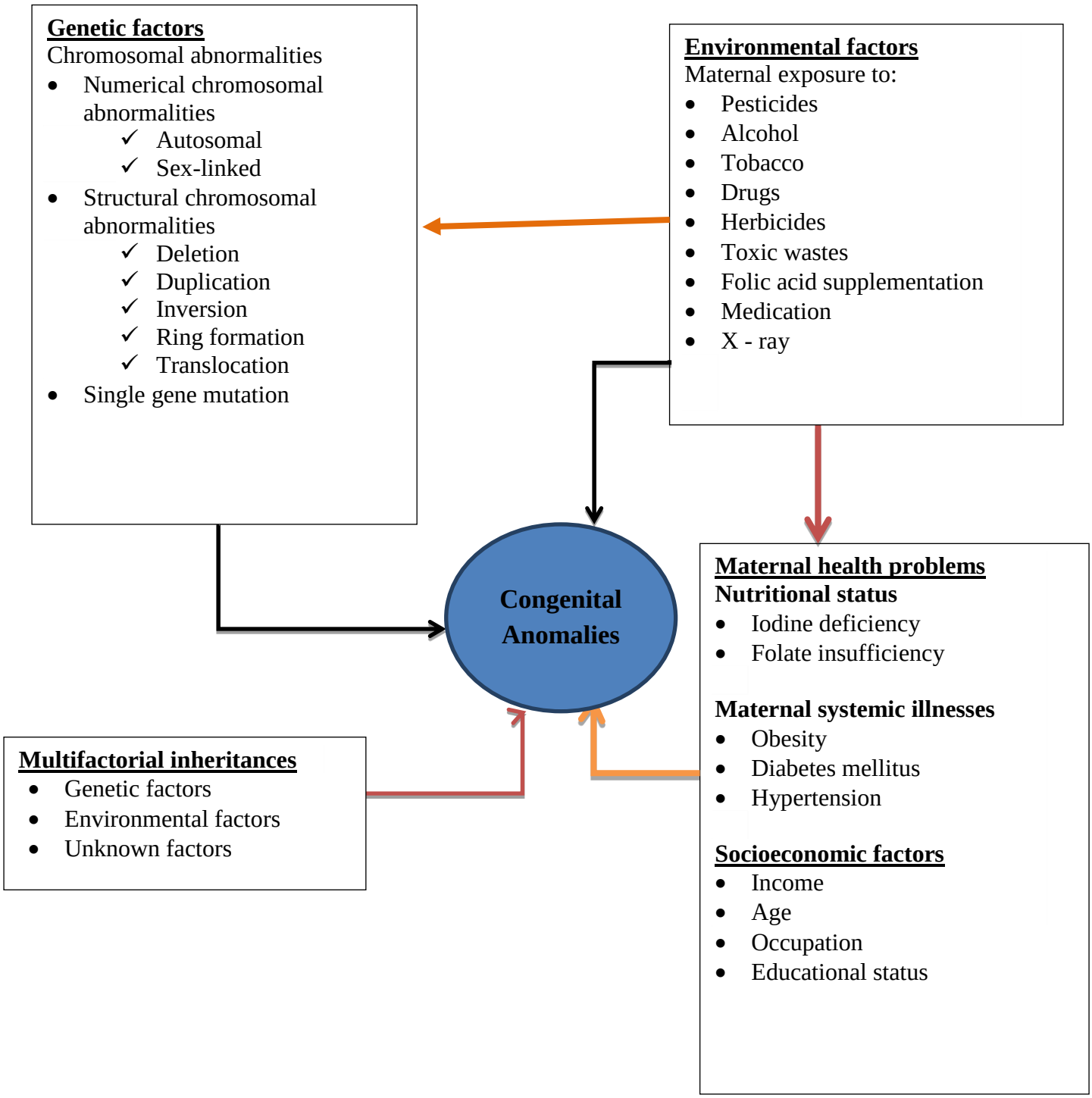
Multifactorial inheritance, is responsible for several developmental disorders resulting in CAs (Graham et at., 2005). Most NTDs are supposed to have a multifactorial inheritance as human development depends on genetic and environmental factors like folic acid suplmantations (Graham et at., 2005).

2.3 Conceptual framework

Existing literature revealed that several decisive overlappings or interrelated factors contribute to the abnormal development of human embryos with which babies born with defects. These included: Genetic, environmental, multifactorial and unknown factors. Based on these contributing factors pertaining to the occurrence of CAs, conceptual framework needed to develop for the present study as described below.

1. **Genetic factors:** Gene is a hereditary factor that can be transferred from parents to the offsprings, constituting the genetic makeup of the offsprings and influence the developmental processes of the newborns at a molecular level. The genetic causes of CAs can be from chromosomal abnormalities (structural or numerical abnormalities) or single gene mutation.
2. **Environmental factors**: Environmental factors largely contribute to the development of CAs. Maternal exposure to environmental factors such pesticides, drugs, alcohol, radiations, tobacco, fertilizers, contaminated water and other hazardous chemical substances during embryonic period/early pregnancy interfere with normal developmental processes leading to abnormal cytogenesis, histogenesis, organogenesis and morphogenesis.
3. **Multifactorial inheritances:** Combination or interactions of genetic factors and environmental factors, where combination of genes from both parents are affected by adverse environmental factors.
4. **Maternal factors:** Maternal factors such as age, nutritional status, illnesses and socioeconomic factors can contribute to the development of CAs in one or another way.

Schematic presentation of conceptual framework



2.4 Research questions

The present work focused on the incidence, magnituied, patterns and a wide range of predisposing factors associated with CAs.

The core research content addressed the following questions:

1. What are the incidences and prevalences of CAs in southwestern Ethiopia?
2. What are the associated risk factors that contributed to the occurance of CAs?
3. What are the predisposing risk factors for the most prevalent CAs?
4. What are the genetic predisposing factors among mothers of the newborn with CAs?

2.5 Objectives of the study

2.5.1 General objective

- To assess the prevalence, incidence, types, and patterns of CAs or disorders at birth and associated risk factors for the malformations in southwestern Ethiopia.

2.5.2 Specific objectives

- To determine the prevalence of different types of CAs at birth in southwestern Ethiopia.
- To determine the incidence of the two years period of CAs at birth in southwestern Ethiopia.
- To identify the possible associated factors for the occurrence of CAs in southwestern Ethiopia.
- To identify the genetic disorders among mothers of newborns with CAs in southwestern Ethiopia.

Chapter Three

3. Subjects and methods

3.1 Study setting

The study was conducted in southwestern Ethiopia - within Oromia regional state. Oromia regional state is the largest region among the ten regions of Ethiopia followed by Amhara regional state. The Oromia regional state is the largest and most populous state in the country and primarily consists of the Oromo ethnic group, the largest ethnic group in the country. Oromia is located in northern, southern, eastern, western and central parts of the country with a total surface area of 286,612 Km^2 (110,662 sq mi). The capital city of Oromia regional state is Finfinne (Addis Ababa), which is also the federal capital city of the country. The Oromia regional state has 20 administrative zones and 280 woredas, and 6500 kebeles. According to the 2011 census , the population of Oromia was estimated to be more than 35,000,000 constituting one-third of the total population of the country.

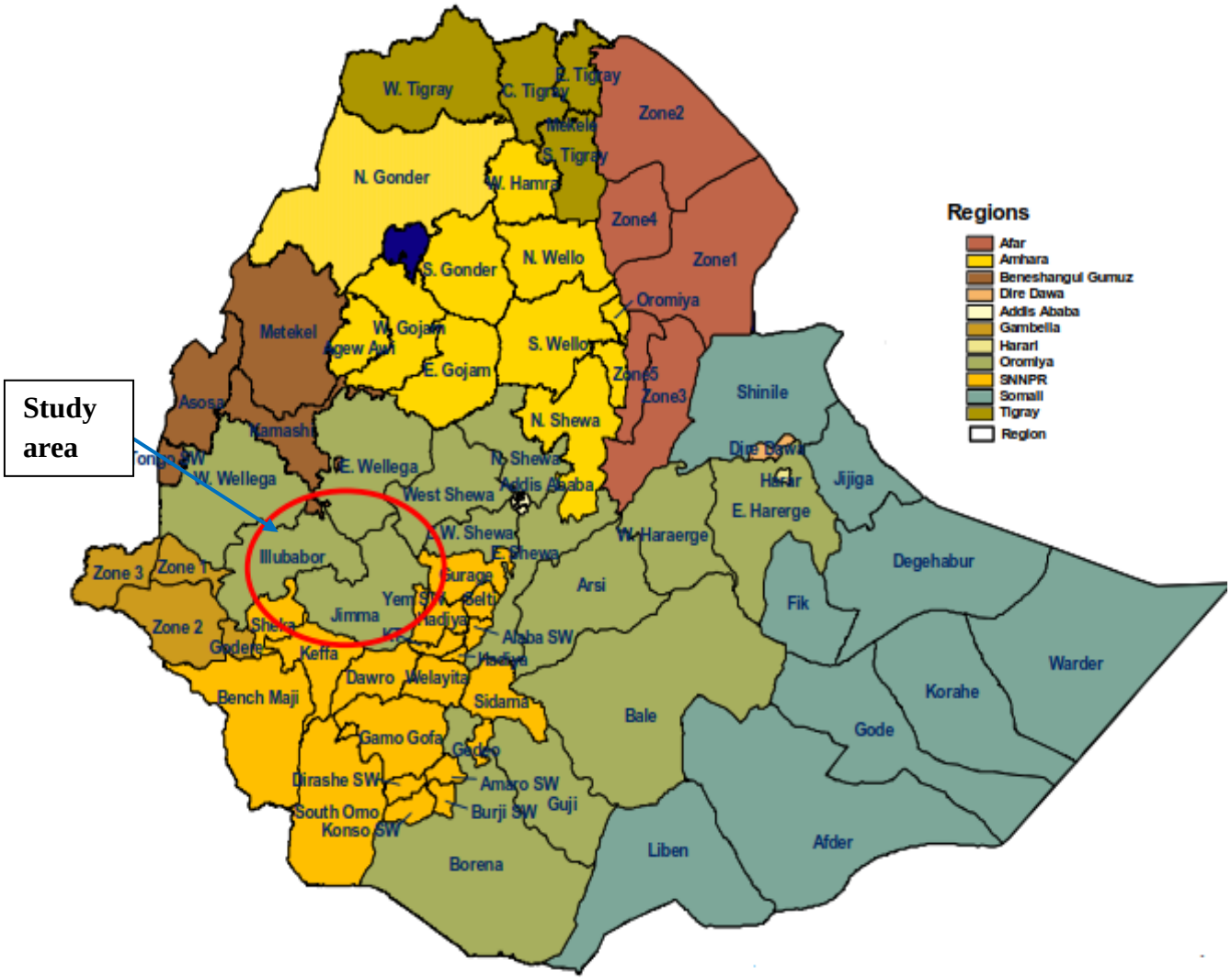


Figure 3:1 Map of Ethiopia showing the study area

The study was conducted in six purposively selected hospitals, on bases of their case load, of the three zones in southwestern Ethiopia, specifically within the Oromia region, which are Jimma, Ilu Abba Bor and east Wollega zones. These three zones are among the seven zones in the western Oromia regional state. Jimma University specialized hospital-currently named as Jimma Medical Centre, Shanan Gibee, Agaro and Limu Genet hospitals are in Jimma zone; Bedele and Metu Karl hospitals are in Ilu Abba Bor zone; Nekemte referral hospitals belong to east Wollega zone. Bedele hospital from Ilu Abba Bor zone was excluded from the study site as it is a newly opened hospital with low capacity and low caseloads. Therefore, this study was carried out in six selected hospitals from these three zones in southwestern Ethiopia namely: Jimma University Specialized, Shanan Gibe, Limmu Genet, Mattu Karl, Agaro and Nekemte hospitals.

3.2 Study design

This research used quantitative epidemiological and laboratory (experimental) study design. The quantitative study design included institution based retrospective cross-sectional, prospective cross-sectional, and case-control. The descriptive cross-sectional study design was used for the incidence and prevalence; a case-control study for the risk factors in selected hospitals in southwestern Ethiopia of the Oromia regional state. In case-control, cases were births (still or live) with CAs while controls were births (still or live) without any CAs.

Retrospective crosses sectional study design: Institution- based retrospective cross-sectional study design was conducted in six purposively selected hospitals with high caseloads from the eight available hospitals in southwestern Ethiopia. This study design involved inspection and review of medical records, to determine the prevalence and the types of CAs . The sampling frame included all deliveries attended in selected hospitals in the southwestern region of Ethiopia from September 2011 to August 2015 during which a total of 45,951 deliveries were attended in the hospitals. The sampling population involved all deliveries with CAs that included both live and stillbirth.

Prospective cross-sectional study design for primary data collections: Caseload based descriptive cross-sectional study design was carried out in six purposively selected referral hospitals in southwestern Ethiopia from May 2016 to May 2018 during which 35,080 delivers

were recorded. The minimum sample size required was 35,080 based on NTDs prevalence estimate of 0.6 extrapolated from the report of two teaching hospitals in Addis Ababa with a confidence interval of 95% and error set at 5% (Gedefaw et al., 2018).

Case-Control: The case-control study design was used to investigate the exposure status of associated risk factors for CAs in case and control newborns (alive and stillbirth) in six selected hospitals in southwestern Ethiopia. Cases were births (still or alive) with minor and major CAs, while the controls were births (fresh still or alive) without any CAs. 251 cases and 887 controls were involved with a total sample size of 1138.

3.3 Source and study population

The source populations for retrospective document review study included all deliveries attended in the selected hospitals in the southwestern region of Ethiopia during the period of 2011 - 2015. The study population involved all deliveries with CAs that included both live and stillbirth.

The source population for the prospective cross-sectional study included all new births and their corresponding mothers who had given birth in a sampled hospital in southwestern Ethiopia. Prospective registration of all deliveries of any gestational age during the study period was collected to determine the incidence of CAs. Cases were all births (still or live) with CAs and mothers who had given birth to the neonates with CAs.

Case-control: Source populations for cases were all births (still or live) with CAs and mothers who had neonates with CAs in the selected hospitals. Source populations for controls were all births (still and live births) without any CAs and their corresponding mothers in the selected hospitals. All the still and live born neonates in the selected hospitals during the study period were included in the study.

Inclusion criteria:

Cases

- All births (live or still) either singletons or multiple at all terms of gestational age with any congenital disorders or CAs.

Controls

- All births (live or still) either singletons or multiple at all terms of gestational age without any congenital disorders, but in the same geographical location with the cases.

Exclusion criteria:

- Mothers whose original ethnic groups are outside the study population
- Non – Ethiopian mothers
- Unconscious mothers in the delivery room
- Unwilling subjects to participate in the study

3.4 Selection of study hospitals

They were seven functional governmental hospitals in the study area, southwestern Ethiopia. All hospitals were purposively included except one, Bedele hospital in Illu Abba Bora zone, in the study after reviewing the hospital record for CAs and capacity to assess the defects among the newborns at their delivery wards. Then case and controls were selected consecutively. Besides, all the selected hospitals have specialties, departments and units that were capable of conducting advanced investigations and provide an organized health care system. All the study hospitals have well-equipped materials with enough instruments that can prove advanced investigations.

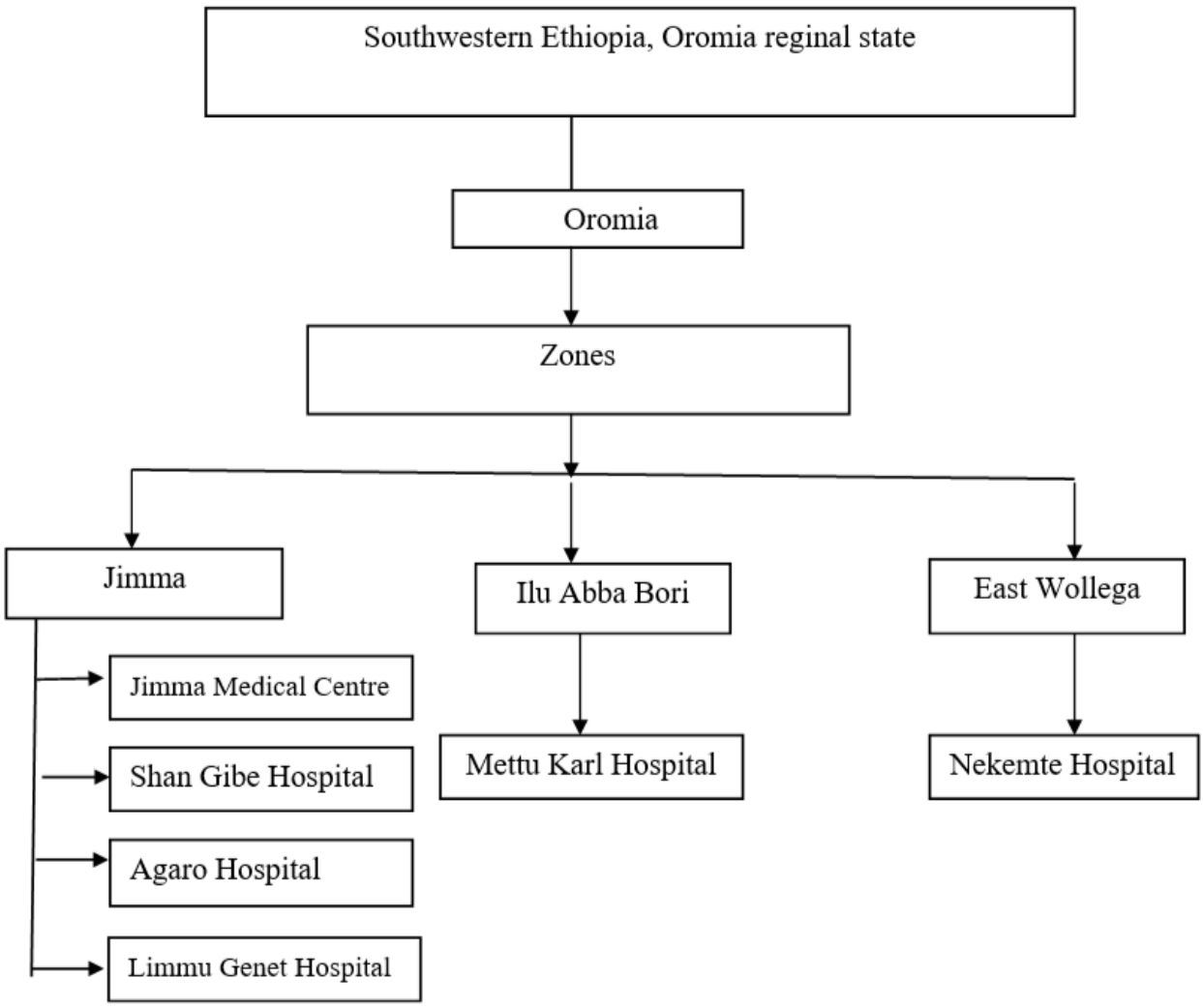


Figure 3:2 Schematic presentation of the study hospitals in southwestern Ethiopia

3.5 Sample size determination

The sample sizes for the different objectives were separately determined. The sample size for the retrospective study was all the medical records of the neonates with CAs in all study hospitals of southwestern Ethiopia from September 2011 to August 2015. The overall sample size was 253 births with CAs.

A total 44,991 record was the minimum sample size estimated according to Dean et al., 2013 with the following assumptions: proportion (p) of CA in Ethiopia to be 20.0 per 1000 birth, confidence interval 95%, degree of precision (d), 0.013%. The absolute precision was reduced from the conventional 5% as the CAs are an infrequent event. To get the required sample, five years' medical records were reviewed, which was a bit greater than the estimated sample size. The sample size for a case studies or case series prospective cross-sectional study was all deliveries with CAs (case/cases) during the study period. The sample size for this part was 251 newborns.

The sample size for the unmatched case-control study assumed a ratio of 1:4, where 251 cases and 887 controls were used with a total sample size of 1138. The sample size was calculated using the case-control ratio of 1:4. The proportion difference approach used with the desired confidence level of 95%, 80% power, and a 5% significant level, assuming that the Odds ratio is expected to be 2 (Kelsey, 2012). Whenever a case occurred, four controls were randomly selected from the existing newborns without CAs at the maternity ward immediately after the occurrence of the case/cases were noticed. Cases were newborns with any major or minor CAs and the mothers who had newborns with CAs.

Obstetrician confirmed the diagnosis of the cases. Types of anomalies were recorded immediately. In the same manner, controls were newborns without CAs and were selected randomly from the newborns at delivery wards where two were selected from normal deliveries before the case and two were selected after the case.

3.6 Data collection methods

Retrospective record reviews data collection methods

All newborn babies were routinely screened for CAs immediately after birth and just before discharge from the hospitals. Diagnosis was usually via physical examinations, then the midwifery staff on duty carried out the discharge as per the order and record of the information on the nationally developed registration logbook. Data were collected with the help of a standard checklist developed by reviewing different previous related data collection instruments. Accordingly, the card number of newborns with CAs were taken from the logbook and sought the card from the card room and reviewed according to the standard checklist prepared for data collection with the supervision of the primary investigator and supervisors. Data were collected by trained midwives, nurses, and general practitioners. For a child born with minor or major CAs, types of CAs, socio-demographic variables such as age, educational status, income, residence, the religion of the mother and obstetric history such as parity, gravida, history of abortion and stillbirth, gestational age, birth weight were retrieved just to characterize the newborn baby.

Prospective data collection methods

Newborns either still or live births in the selected hospitals during the study period were evaluated for the presence of any CAs by trained health professionals. Standard checklists were used to assess every live and stillbirth. CAs were assessed by trained residents, general practitioners, and midwives at delivery wards in the study hospitals. The trained midwives seriously followed whenever suspected cases of CAs were delivered and data were collected before the discharge of the mother within the 24 hours of the delivery. Cases were confirmed by Obstetrician working at the hospitals thorough physical examination and some radiological investigations. Any anomalies or defects identified were described and recorded. Prior to data collection, the respective mothers were informed about the aim of the study and reached the consensus to participate (both cases and controls). The data were collected from the mothers using structured questionnaire containing variables regarding socio-demographic, socioeconomic, reproductive and obstetrics, maternal characters and neonate characters such as birth weight, birth order, sex of the newborn, birth outcome and types of birth outcome.

Case-control

Newborns, either stillbirth or live births were evaluated for the presence of any CAs. A standard pretested checklists were used to assess every live and fresh stillbirth. The assessment of CAs was conducted by trained residents, general practitioners, and midwives at Obstetrics and Gynecology delivery wards. The data were collected from the mothers of cases and controls using a structured questionnaire containing variables regarding socio-demographic, maternal characters, neonate characters, and associated risk factors.

3.7 Study variables

1. **Dependent variables**
   - Congenital anomalies
   - Types of congenital anomalies
2. **Independent variables**
   - Maternal age
   - Socioeconomic status
   - Educational background
   - Family history
   - Maternal factors: Cigarette smoking, passive smoking, alcohol drinking, drug abuse, unidentified drug usage during early pregnancy.
   - Water sources for drinking
   - Maternal health status (medical disorder),
   - Maternal exposure to drugs
   - Maternal exposure to infection
   - Maternal exposure to chemicals and pesticides, medications, Khat and waste disposal areas
   - Mode of deliveries, gestational age, sex and birth weight of the neonate.
   - Maternal exposure to radiation
   - Maternal chronic disease: Diabetes millets, hypertension, asthma and other disorders
   - Maternal antenatal care follow up
   - Maternal folic acid supplementation

3.8 Operational definitions

Birth defect: Developmental abnormality present at birth.

Congenital anomaly: Wide range of abnormalities of body structure or function that are present at birth and are of prenatal origin.

Major Anomaly: Developmental abnormalities that have surgical or medical significance leading to postnatal morphological defects.

Minor anomaly: A developmental feature that varies from normal structure but does not bring functional abnormalities.

Neural tube defects: Congenital anomalies related to nervous system.

Malformation: A morphologic defect of an organ, part of an organ, or larger region of the body that results from an intrinsically abnormal developmental process.

Genetic disorder: A disease caused in whole or in part by a change in the DNA sequence away from the normal sequence.

Disruption: A morphologic defect of an organ, part of an organ, or a larger region of the body that results from the extrinsic breakdown of, or an interference with, an originally normal developmental process.

Deformation: An abnormal form, shape, or position of a part of the body that results from mechanical forces.

Dysplasia: An abnormal organization of cells into tissue(s) and its morphologic result(s).

Prevalence: Statistical concept referring to the number of cases of a disease that are present in a particular population at a given time.

Incidence: Number of new cases that develop in a given period of time.

Risk factor: Factors responsible for the occurrence of abnormal embryonic developing

Teratogen: Any environmental factor that causes abnormal development of the embryo/feteus.

3.9 Data quality control

The questionnaires were prepared in English then translated into Amharic and Afan Oromo and back-translated into the English language by another person to check the semantic equivalence. It was pre-tested on 5% of the sample size in the non-selected health center

districts. Completed questionnaires were checked for consistency and completeness by the principal investigator.

The result of the research will be communicated to Addis Ababa University and Jimma University, CDT-Africa and to the community, presented on scientific conferences, published on national or international journals.

3.10 Data processing and analysis

Retrospective record review

The collected data were cleaned, entered to Epi Data computer software and analysed using SPSS software program version 22.0. The outcomes of fetal birth defects were determined intermesh of mode of deliveries. Birth defects were classified using the International Classification of Diseases (ICD-10). Total prevalence has been calculated by dividing the numerator (registered cases of CAs) by the relevant denominator (total live and stillbirths) for the same period of time at the same place. Infances with more than one anomalies were counted as multiple defects based on the available primary diagnosis. A P-value of less than 0.05 was considered as statistically significant.

Prospective registry and case - control

The collected data were checked regularly on daily basis, by trained data collectors and further superintended by the principal investigator and supervisors. The collected data were cleaned, coded, and entered into Epi Data computer software and transferred to SPSS version 25.0 for analysis. The outcome of fetal birth disorder was determined intermesh of mode of deliveries. CAs were classified according to the International Classification of Diseases. Total prevalence was calculated by dividing the numerator (registered cases of CAs) by the relevant denominator (total live and stillbirths) for the same period of time at the same place. Overall data was calculated using descriptive statistics such as frequency, cross-tabulation, and logistic regression. A P-value of less than 0.05 was considered statistically significant.

Crud Odds ratios (COR) and confidence intervals were calculated to assess crud risk estimates for the risk factors. From the computed COR, Adjusted Odds ratios (AOR) were calculated to

investigate possible risk factors (predictors) associated with the occurrence of CAs by using a multivariant logistic regression model. Exposure variables with P-value ≤0.2 in COR were entered into a multivariable logistic regression model to evaluate the association between the exposure and CAs. Maternal and neonatal characteristics of the cases and the controls were presented in number and percentage. COR and AOR, confidence intervals, and P-values as well as the results of the findings are presented in the form of text and Table(s).

3.11 Laboratory based study (Expermental Part)

Study Area: Jimma University, Molecular Biology Lab
Addis Ababa University, School of Medicine, Core Lab
MRC – ET, Advanced Laboratory, Addis Ababa

3.11.1 Sample size determination

Scientific studies described that the commonest sample size for genomic or genetic studies are varying from 25 – 60 samples based on the availability and effectiveness of genomic analysis method used and funding opportunities. According to Hale et al. (2012), the most effective sample size for the genomic study was 25 – 30 per population. Besides, Jankowski et al. (2008) indicated that a copy number variation analysis for 50 DNA samples has been successfully demonstrated using MLPA assays technology. In the present study, 20% of a total sample of 251 were used (251*20% = 50.2 sample). Hence, 50 DNA samples weres used for the genomic analysis with help of MLPA assay which can perform up to 50 DNA sample in one reaction efficiently in this study.

3.11.2 Sample collection

3.11.2.1 Blood sample collection

8-10 ml of perripheral blood were collected from mothers of neonates born with CAs using standard blood draw in carefully labeled EDTA or heparin tubes by trained health professionals after taking consent from the study participants. The blood samples were immediately placed on rocker and allowed to mix for more than 10 minutes to avoid the formation of micro clots and were stored in refrigerator at negative 70^{o}C until processed.

3.11.2.2 Quality control and safety issue

During sample collection and processing, all biohazard safety guidelines and regulations including universal precautions for handling human specimens were followed. Biological samples were handled under certified biological cabinet.

3.11.2.3 Blood DNA extraction and isolations

DNA was extracted from peripheral blood taken from the patients and kept frozen at -20 degree celcius using the DNeasy blood and tissue DNA extraction kit (Qiagen, www.qiagen.com) according to manufacturers instruction. 200 µl of anticoagulant treated blood sample was taken from the EDTA tube and transferred to 1.5 ml microcentrifuge tube. 20 µl proteinase K was added into the 1.5ml micro centrifuge tube containing blood sample. 200 µl buffer AL was added and mixed thoroughly by vortexing. Then incubated in an incubator at 56^0C for 3 hours. 200 µl ethanol (96 – 100%) was added and mixed thoroughly with vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collecting tube and centrifuged at ≥6000 x g (12,000 rpm) for 1 min. The flow-through and the collecting tube were discarded and the spin column was placed in a new collecting tube. 500 µl Buffer AW1 was added and centrifuged for 1min at ≥6000 x g, and the flow – through and collection tube discarded. The spin column was then placed in a new 2 ml collection tube and 500 µl Buffer AW2 was added and centrifuged for 3 min at 20,000 x g (14,000 rpm). The flow-through and the collection tube were discarded. The spin column containg tube were centrifuged empty at 20,000 x g (14, 000rpm) for 1 min to eliminate ethanol. Finally, the spin column was transferred to a new 1.5 ml microcentrifuge tube and 100 µl Buffer AE was added to the center of the spin column membrane to elute the DNA and then incubated for 1 min at room temperature (15 – 25^0C). Finally it was centrifuged for 1 min at ≥6000 x g. This was repeated 2 times to increase the DNA quality and yield.

3.11.2.4 Determination of DNA quality and quantity

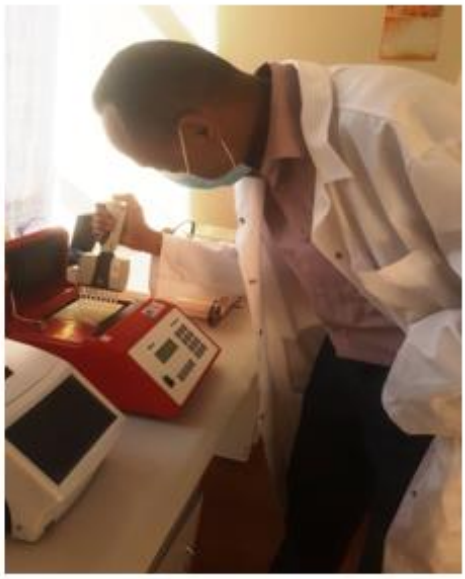
Once DNA isolation was completed, the concentration and purity of the resulting DNA was measured with the use of a nanodropspectrophotometr (WPA,Cambridge, UK) and the purity of the DNA was determined by using ratio of A260nm/A280nm estimation prior to the reading of DNA samples. TE buffer measured at a 260/A280 nm was used as a blind.

Pure DNA at A260/A280 nm ratio in sample without protein contamination is reaching very close to 1.6; A260/A280 mn ratio is lower than 1.6 in the presence of faults caused by the solutions and proteins used in isolation. The concentration of DNA samples was determined as ng/l. Nano drop quantification of DNA samples with spectrophotometer resulted in 100ng/l concentrations for both polymorphisms and its concentrations were adjusted by TE buffer. The DNA samples were used immediately for PCR or placed at negative 80^0C for long term storage.

a. Lebeling samples

b. Adding DNA extraction Kits

c.Centrfuguation of extracted DNA materials



d. Adding sample for MLPA processes

Figure 3:3 Pictures showing some activities done during sample preparations for MLPA analysis

3.11.3 MLPA genomic anlysis

3.11.3.1 Preparation of samples

DNA Denaturation

Strips or plates of 0.2ml were labeled. 5 µl DNA sample (50 – 100 ng) was added to each tube. Produced female and male sample were used as controls. The tube containing DNA samples were placed in thermocycler followed by the start of MLPA thermocycler program. The DNA samples were denaturized at 98^0C for 5 min. Then cooled to 25^0C before removing from thermocycler.

Hybridization

Hybridization master mix for each probe mix (MLPA kit P070 and Salsa MLPA buffer, MRC-Holland Bv. Amsterdam, the Netherlands) containing 1.5 µl MLPA buffer and 1.5 µl probe mix was made ready before use. 3 µl of the hybridization master mix was added to each sample tube and mixed well by pipetting up and down, then incubated for 1 min at 95^0C. The hybridization process continued for 16 – 20 hours at 60^0C.

Ligation

At this stage, the DNA target sequences that are completely hybridized the MLPA probes that are adjacent to each other and are joined by an enzyme called ligase. The two ligase buffers (buffer A and buffer B), (MRC-Holland Bv. Amsterdam the Netherlands) were vortexed prior to use. A ligation reaction master mix was prepared for each reaction by adding 3 µl Ligase buffer A plus 3 µl ligase buffer B and mixed with 25 µl distilled water (dH_2O). Then 1 µl Salsa ligase–65 enzyme (MRC-Holland Bv. Amsterdam the Netherlands) was added and mixed well by pipetting gently up and down. The thermocycler program continued but paused at 54^0C for the overnight hybridized samples to reach at 54^0C. Then 32 µl Ligase mster mix was added to each tube and mixed gently. The thermocycler is programmed to continue with a 15 min incubation at 54^0C (for ligation); then 5 min at 98^0C for heat inactivation of Ligase- 65 enzyme followed by apause at 20^0C to remove the tubes from thermocycler.

PCR Amplification:

The PCR reaction was prepared by first mixing the SALSA PCR primer mix (MRC-Holland Bv. Amsterdam the Netherlands) by vortexing and warming the Salsa polymerase (MRC-Holland Bv. Amsterdam the Netherlands) for 10 sec in hand to reduce viscosity. Polymerase

master mix was prepared by adding 7.5µl dH_2O plus 2 µl Salsa PCR primer mix plus 0.5 µl Salsa polymerase and mixed well by pipetting up and down and used for the next step or stored in ice. 10 µl polymerase mix was added to each tube at room temperature and then mixed. Continuing the PCR on the thermocycler with a program profile: 35 cycles of 30 seconds at 95^0C; 30 seconds 60^0C; 60 seconds 72^0C, ended with 20 min final extension at 72^0C. After the cysle is completed, the reaction paused at 15^0C. Following the PCR reaction or amplification, the tubes remained closed in the room with thermocycler to avoid contamination. We used different micropipettes for performing MLPA reaction and handling MLPA PCR products. 1 µl of the PCR products were used for fragment analysis or sequencing by capillary electrophoresis. For comparative purpose, gel electrophoresis were also used to detect the quality of the PCR amplifications for the next step. Thus, the quality of the PCR reactions (amplifications) was determined by agaros gel electrophoresis for the final MLPA analysis or fragment analysis with the help of capillary electrophoresis (Figure 4).

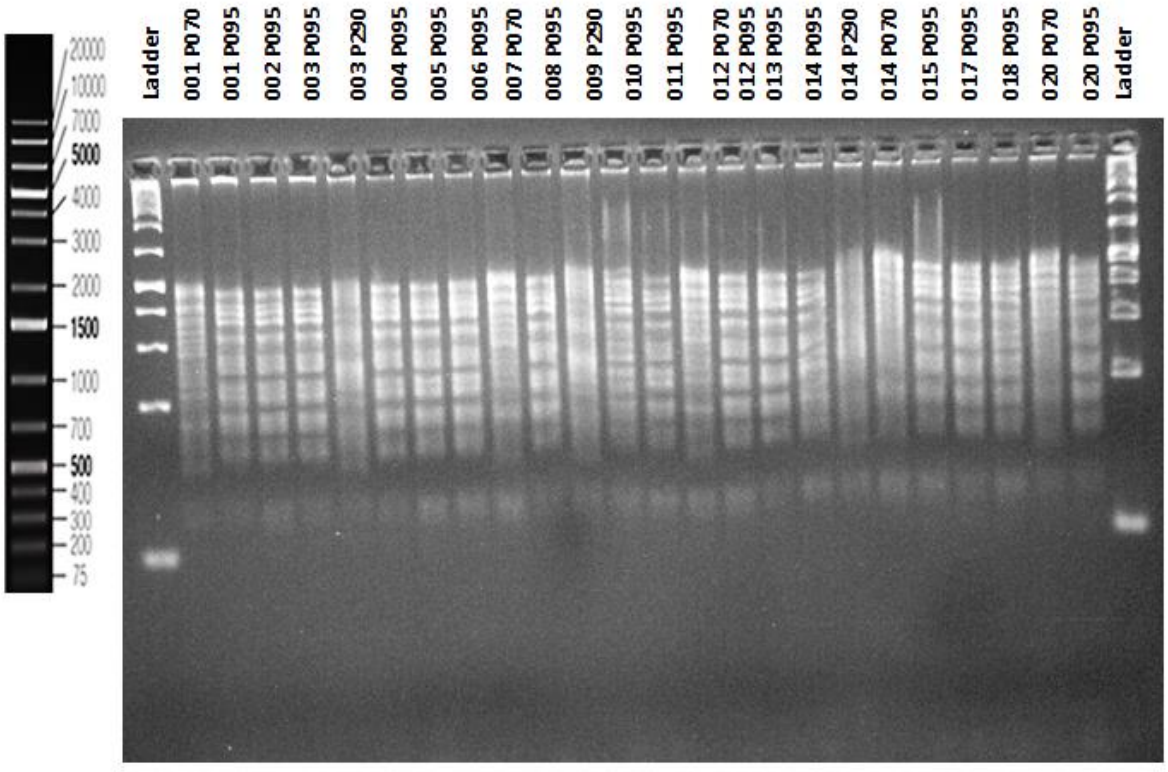


Figure 3:4 Electropherogram of agaros gel electrophoresis of randomly selected PCR mplified genomic samples showing segements of nucleotides

Figure 4: Electropherogram of agaros gel electrophoresis of randomly selected PCR amplified genomic samples showing segements of nucleotides.

3.11.4 Data analysis

The DNA fragments were separated by size using capillary electrophoresis. The data were analyzed by using Coffalyser fragment analyzing software, Version: v.140721.1958 (MRC-Holland Bv. Amsterdam the Netherlands), with probe final ratio range of 0.7 to 1.3. The result of comparative analysis was determined. The relative peak area for each probe was calculated as a fraction of the total sum of peak areas in a given sample. Each autosomal peak fraction was divided by the median peak fractions of that locus for all samples in the reaction. The relative probe signal values (final ratio) between 0.7 and 1.3 in comparative MLPA analysis were defined as normal. The absence of signal or the value of 0 is considered deletion, the value of target sequence < 0.7 was considered to be heterozygous deletion, whereas the target sequence value >1.3 was considered to be duplication.

3.12 Ethical consideration

Ethical approval was obtained from Addis Ababa University, College of Health Sciences, Institutional Review Board, meeting Ref. No. 005/16, dated May 2016. Supportive letters were submitted to all study hospitals' administrators. Then data collections were stared after permissions were granted from the medical directors of each hospital. The study procedure and its aim were disclosed for participants. All the study participants were informed that participation is voluntary based and can be withdrawn at any time. Not volunteering did not affect the servces that they did get from the hospital. Written and signed consent was obtained from all study participants before administering the structured questionnaires. The participants did not write their names on the questionnaires for confidentiality and no identifying information was recorded by participants. The data were coded, stored in a safe and secure location. The data were never exposed to anyone and only used solely for the study purpose.

Table 3:1: Summary of the study for manuscript preparation (1 – 5)

No	Objectives	Study design	Study subject	Sample size with CAs	Data collection methods	Data analyzing methods
1	To determine the prevalence of CAs	Retrospective Cross sectional Document review	Newborns, Mothers of the newborns	253	Structured check lists/ questionnaire	Descriptive statistics
2	To determine the incidence of CAs and associated maternal factors	Prospective Cross sectional	Newborns, Mothers of the newborns	251	Interview, Structured questionnaire	Descriptive statistics
3	To evaluate the predisposing risk factors of CAs	Case – control	Newborns, Mothers of the newborns	1138	Interview Structured questionnaire	Inferential statistics
4	To identify the most prevalent CAs and associated factors	Case – control	Newborns, Mothers of the newborns	885	Interview Structured questionnaire	Inferential statistics
5	To determine the genetic risk of CAs	Genomic analysis (MLPA)	Mothers of the newborns with CAs	50	Experimental	Genetic Analysis

Chapter Four

4. Results

4.1 Objective one: Prevalence and patterns of congenital anomalies

From 2011 to 2015, a total of 45,951 deliveries were recorded. Out of these, 253 births were with CAs. This makes the overall prevalence of CAs 5.5 per 1000 births. The majority (92%) of the mothers were younger than 35 years indicating that factors responsible for the defects were might not be related to maternal age. About half of the defects (49.6%) occurred among mothers whose ages were between 25 - 35 years. 51.38%, 48.22% and 0.4% of children born with CAs were males, females and ambiguous genitalia, respectively by their sex. About 63.1% of neonates with CAs were born as stillbirth. The majority 92.5% and 93.3% of the mothers had no previous history of abortion and stillbirth, respectively (Table 4:1).

Table 4:1: Socio-demographic and obstetric characteristics of mothers and newborns with birth defects from 2011 to 2015 (n = 253)

Characteristics		Frequency	%
Religion of the mothers	Muslim	115	45.6
	Orthodox	33	13.1
	Protestant	26	10.3
	Not mentioned	78	31.1
Age of the mothers	15-24	105	41.7
	25-35	125	49.6
	36-43	12	4.8
	Missing	10	4.0
Onset of labor	Spontaneous	202	80.2
	Induced	50	19.9
Mode of delivery	Vaginal	213	84.5
	Caesarean section	39	15.5
Sex of the newborn	Male	130	51.4
	Female	122	48.2
	Ambiguous genitalia	1	0.4
Status at birth	Alive birth	93	36.9
	Stillbirth	159	63.1
History of abortion	Yes	19	7.5
	No	233	92.5
History of stillbirth	Yes	17	6.7
	No	235	93.3
Gestational age	Term	139	55.2
	Preterm	99	39.3
	Post term	3	1.2
	Missing	11	4.4
Birth order	1-4	116	46.0
	5-9	41	16.3
	Missing	95	37.7

Out of four major different types of BDs identified in this study (Table 3), NTD was the most prevalent with the frequency of 73.5% followed by gastrointestinal defects (13.4%) and musculoskeletal defects (11.1%). Genitourinary defects were the least prevalent with the frequency of 2% (Table 4:2).

Table 4:2: Frequency of CAs by sex among deliveries from 2011 to 2015

Birth defects	**Male (n = 130)**		**Female (n = 122)**		**Total (n= 253)**	
	Frequency	**%**	**Frequency**	**%**	**Frequency**	**%**
Neural tube defects	89	67.9	97	79.5	186	73.5
Gastrointestinal defects	20	15.3	14	11.5	34	13.4
Musculoskeletal defects	17	13.0	11	9.0	28	11.1
Genitourinary defects	4	3.1	0	0	4+ 1* =5	2.0

1* ambiguous genitalia

Of the NTDs recorded, anencephaly (33.9%) and hydrocephalus (33.3%) were the most frequent followed by spina bifida (17.7%). Microcephaly (1.6%) and craniorachischisis (1.6%) were the least frequent. As shown in Table 4:3, females were more affcetd with NTDs than males with frequency of 52.2%. Specifically, however, more females were affected with anencephaly and spina bifida with the frequence of 20.43% and 9.68%, respectively. Whereas, more males were affected with hydrocephalus, meningomyelocele, microcephaly and craniorachischisis with the frequency of 17.2%, 5.37%, 1.61% and 1.1%, respectively.

Table 4:3: Frequency of NTDs among deliveries from 2011 to 2015 in southwestern Ethiopia

Neural tube defects	Male (n=89)		Female (n = 97)		Total (n = 186)	
	Frequency	%	Frequency	%	Frequency	%
Anencephaly	25	28.1	38	39.2	63	33.87
Hydrocephalus	32	17.2	30	30.9	62	33.33
Spina bifida	15	16.9	18	18.6	33	17.74
Meningomyelocele	10	11.2	8	8.3	18	9.67
Encephalocele	2	2.3	2	2.1	4	2.15
Microcephaly	3	3.3	0	0	3	1.61
Craniorachischisis	2	2.3	1	1.03	3	1.61

Among gastrointestinal defects, the percentage of congenital umbilical hernia, imperforate anus, gastroschisis, duodenal atresia, congenital inguinal hernia and omphalocele was 35.3%, 26.5%, 17.7%, 8.8%, 8.8% and 2.9%, respectively (Table 4:4). Umbilical hernia was the most prevalent (35.3%) and omphalocele was the least (2.9%).

Table 4:4: Frequency of gastrointestinal defects among deliveries from 2011 to 2015 in southwestern Ethiopia

Gastrointestinal defects	Male (n=21)		Female (n = 13)		Total (n = 34)	
	Frequency	%	Frequency	%	Frequency	%
Umbilical hernia	3	14.3	9	69.2	12	35.3
Imperforate anus	7	33.3	2	15.4	9	26.5
Gastroschisis	5	23.8	1	7.7	6	17.7
Duodenal atresia	2	9.5	1	7.7	3	8.8
Congenital inguinal hernia	3	14.3	0	0	3	8.8
Omphelocele	1	4.8	0	0	1	2.9

Among the musculoskeletal defects, the proportion of clubfoot, cleft lip, cleft palate, both cleft lip and palate and chest deformity were 36%, 24%, 20%, 16% and 4%, respectively in decreasing order of their frequencies (Table 4:5).

Genitourinary defects were the least frequent constituting 2% of the total major BDs. Among genitourinary defects, the proportion of hypospadias, meatal stenosis, ambiguous genitalia were 50%, 37.5% and 12.5%, respectively. The result showed that hypospadias was the most frequent and ambiguous genitalia were the least frequent.

Table 4:5: Frequency of musculoskeletal defects among deliveries from 2011 to 2015 in southwestern Ethiopia

Musculoskeletal defects	Male (n=15)		Female (n = 10)		Total (n = 25)	
	Frequency	%	Frequency	%	Frequency	%
Clubfoot-bilateral	3	20.0	6	60.0	9	36.0
Cleft lip	4	26.7	2	20.0	6	24.0
Cleft lip and palate	3	20.0	2	20.0	5	20.0
Cleft palate	4	26.7	0	0.0	4	16.0
Chest deformity	1	6.7	0	0.0	1	4.0

Of the twenty-one CAs identified in the present study, five types of CAs namely: anencephaly (25.0%), hydrocephalus (24.6%), spina bifida (13.1%), meningomyelocele (7.1%), and umbilical hernia (4.8 %) accounted about three-fourth (75%) of all recorded CAs (Table 4:6).

Table 4:6: Types and frequency of CAs recorded among deliveries from 2011 to 2015 in southwestern Ethiopia (n= 253)

Types of Birth defects	Frequency	(%)
Anencephaly	63	25.0
Hydrocephalus	62	24.6
Spina bifida	33	13.1
Meningomyelocele	18	7.1
Umbilical hernia	12	4.8
Imperforate anus	9	3.6
Clubfoot-bilateral	9	3.6
Cleft lip	6	2.4
Gastroschisis	6	2.4
Cleft lip and palate	5	2.0
Hypospadias	4	1.6
Cleft palate	4	1.6
Encephalocele	4	1.6
Duodenal atresia	3	1.2
Microcephaly	3	1.2
Meatal stenosis	3	1.2
Craniorachischisis	3	1.2
Congenital inguinal hernia	3	1.2
Chest deformity	1	0.4
Ambiguous genitalia	1	0.4
Omphelocele	1	0.4

The association between the common types of defects and characteristics of the mothers and children were computed using Fisher's Exact Test. Only status of the child at birth (P-value =0001), birth weight (P-value =0.0001) and gestational age (P-value =0.0001) were found to be associated with types of birth defects.

4.2 Objective two: Incidence and prevalence of congenital anomalies

A total of 35,080 deliveries were documented during the study period. Of these, 251 cases of CAs were screened prospectively. About 24 different types of CAs were identified with a total anomalies of 290. Most of the anomalies were single, others were multiple. Most of the CAs (93.5%) were major defects while only 1.4% were minor defects. About 5.1% of the defects associated with complications such as cystic hydroma, and ventriculomegaly were identified and were secondary to the major anomalies and existed with those major defects.

As indicated in Table 4:7, most of the cases were identified in Jimma Medical Center (JMC) with a frequency of 151 (60.2%) and an incidence rate of 43.0 per 10,000 births followed by Agaro hospital 50 (19.9%) with an incidence rate of 14.3 per 10,000 births. The least cases were spotted in Limu Genet hospital with a frequency of 6 (2.4%) and incident rate of 1.7 per 10,000 births followed by Shenen Gibe hospital with a frequency of 8 (3.2%) and incidence rate of 2.3 per 10,000 births. 16 (6.4%) were from the Nekemte referral hospital with an incidence rate of 4.6 per 10,000 births. The overall incidence rate of CAs in southwestern Ethiopia was 71.6 per 10,000 births. The frequency distribution of CAs by sex are shown in Figure 4:1.

Table 4:7: Frequency of study participants with recognized CAs in six selected hospitals in southwestern Ethiopia

S.n	Study hospitals	Cases (CAs)	
		Frequency	%
1	Jimma Medical Center (JMC)	151	60.2
2	Agaro hospital (AH)	50	19.9
3	Limu Genet hospital (LGH)	6	2.4
4	Mattu Karl hospital (MKH)	20	8.0
5	Shenen Gibee hospital (SGH)	8	3.2
6	Nekemte hospital (NH)	16	6.4
Total		**251**	**100**

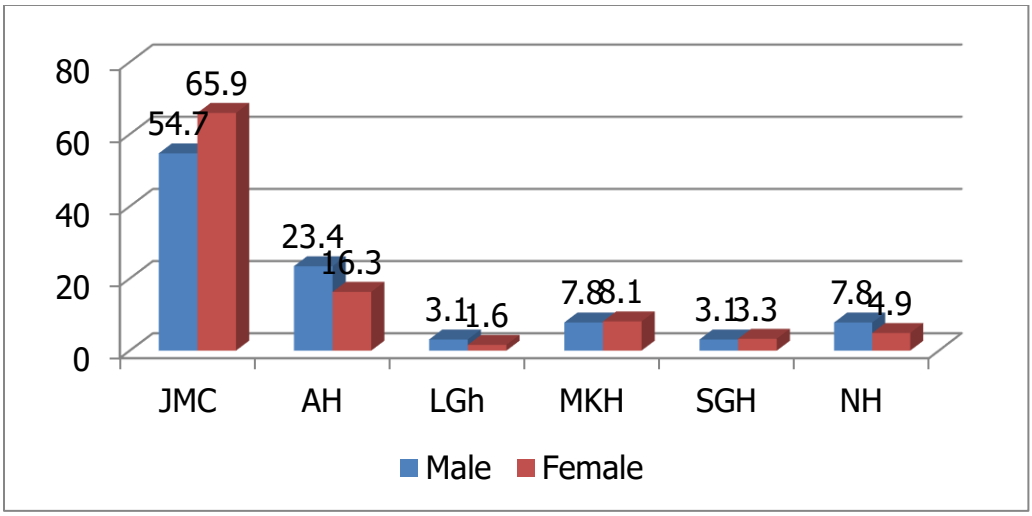


Figure 4:2 Frequency of CAs by sex in study hospitals among deliveries from 2016 - 2018 in southwestern Ethiopia

JMC: Jimma Medical Center; **AH**: Agaro hospital; **LGH**: Limu Genet hospital; **MKH**: Mattu Karl hospital; **SGH**: Shenen Gibee hospital; **NH:** Nekemte hospital.

Socio-demographic characteristics of the study participants in southwestern Ethiopia

The mean age of the mothers who gave birth to newborns with CAs was 25.6 with a range of 17 to 42 years old. About 3.6% of the mothers were above 35 years old, while 32.3% and 37.8% were between 21 – 25 and 26 – 35 years old, respectively. About 52.2% of mothers had a formal education, and the remaining 47.8% had no formal education.

Of the mothers who had a formal education, about 37.4% of them attended their high school level (grades 9 – 12). About 35.1% had junior level (5 - 8) and 9.9% had elementary school (1 - 4) only. About 17.6% of the mothers had atteneded to the tertiary level. Regarding the religion of the study participants, more than half of them, 164 (65.3%) were Muslims, followed by Orthodox christians 48 (19.1%) and the remaining 39 (15.5%) were Protestants.

As shown in Table 4:8, about 151 (60.2%) of the mothers who had neonates with CAs were housewives, and 34 (13.5%) were farmers. 29 (11.6%), 11 (4.4%) and 8 (3.2%) were governmental or NGO employees, merchants, and unemployed, respectively.

About 26.4% of the study subjects had an average monthly income of less than one thousand Ethiopian Birr, while 67.9% of the participants with recognized cases of CAs had an average monthly income between one thousand and five thousand Ethiopian Birr. Only 1.4 % of the participants – the cases, had monthly income great or equal to eleven thousand Ethiopian Birr. 21.9 % of the fathers of the neonates with CAs were < 25 years old. 41.4% of the paternal age was ranging between 26 – 34 years and 33.5% were ≥35 years.

Table 4:8: Socio-demographic characteristics of the study subjects who gave birth to newborns with CAs in southwestern Ethiopia (n = 251)

Characteristics		Frequency	%
Age of the mother in year	<=20	66	26.3
	21 - 25	81	32.4
	26 - 35	95	38.0
	>= 36	9	3.6
Maternal education	Illiterate	120	47.8
	Literate	131	52.2
Maternal educational level	1 -4	13	9.9
	5 - 8	46	35.1
	9 – 12	49	37.4
	13 and above	23	17.6
	Missings	120	47.8
Religion	Muslim	164	65.3%
	Orthodox	48	19.1%
	Protestant	39	15.5%
Average monthly income	< 1000	37	26.4
	1000 -5000	95	67.9
	6000 -10000	6	4.3
	11000 and above	2	1.4
	Misings	111	44.2
Maternal occupation	Housewife	151	60.2
	Farmer	34	13.5
	Employee	29	11.6
	Merchant	11	4.4
	Unemployed	8	3.2
	Missings	18	7.2
Fathers' age	<=25	55	21.9
	26 - 34	104	41.4
	>=35	84	33.5
	Missing	8	3.2

About 78.5% and 20.7% of the onset of labor in case mothers were spontaneous and induced, respectively. 82.9% and 16.7% of the deliveries of the newborns were vaginal and scissoral, respectively. 12.7% of the mothers of the neonates with CAs had a history of abortion and 9.6% of them had a history of stillbirth. 2.8% of the mothers of the cases had a previous birth history of CAs. 3.8% and 4.4% of the mothers and the fathers of the cases had previous history of CAs in their family, respectively. Selected reproductive history of the case mothers is shown in Table 4:9.

Table 4:9: Selected reproductive history of mothers who gave birth to neonates with CAs in southwestern Ethiopia (n = 251)

Characteristics		Frequency	%
Parity	0 & 1	134	53.4
	2 & 3	68	27.1
	>= 4	44	17.5
	Missing	5	2.0
Gravid	0 & 1	90	35.9
	2 & 3	83	33.1
	>= 4	74	29.5
	Missing	4	1.6
Onset of labor	Spontaneous	197	78.5
	Induced	52	20.7
	Missing	2	0.8
Mode of delivery	Vaginal	208	82.9
	Scissoral	42	16.7
	Missing	1	0.4
History of abortions	Yes	32	12.7
	No	216	86.1
	Missing	3	1.2
History of still births	Yes	24	9.6
	No	227	90.4
Pervious history of CA	Yes	7	2.8
	No	244	97.2
History of CA in the family	Yes	11	4.4
	No	236	94.0
	Missing	4	1.6

Of the total CA cases identified, 51.0% were male and 48.6% were females. Of the 251 cases, about 39.8% of the neonates were born with low birth weight (<2500g) and 60.2% were born with normal birth weight (>= 2500g). About 61.8% newborns were stillbirth and 38.2% were alive birth. Stillbirths were the most frequent, indicating that CAs largely affect the birth outcome of the neonate.

Gestational age of the CA cases was classified as preterm (<36 weeks) 47.6%, term (37 – 40 weeks) in 40.8%, and post-term (>40 weeks) in 12%. The preterm was more frequent among classes of gestational age indicating that most CA cases were born before completing their developmental terms. About 80.5% of the newborns with CAs were birth order < 5 for their family and 19.5% had birth order of ≥5 in their family (Table 4:10). 94. 4% and 5.6% of the types of the birth outcome of newborns were singletons and multiple births (twins) respectively.

Table 4:10: Neonatal characteristics of newborns with CAs in southwestern Ethiopia (n = 251)

Characteristics		Frequency	%
Sex of the newborn	Male	128	51.0
	Female	122	48.6
	Ambigous genitalia	1	0.4
Birth weight(Gram)	<2500	100	39.8
	>= 2500	151	60.2
Birth outcome	Alive birth	96	38.2
	Still birth	155	61.8
Type of birth outcome	Single	237	94.4
	Twin	14	5.6
Gestational age at birth (weeks)	Preterm	119	47.6
	Term	102	40.4
	Post-term	29	12
Birth order of the infant	< 5	202	80. 5
	≥5	49	19.5

Maternal responses to wards associated factors for CAs:

As shown in Table 4:11, among mothers who gave birth to the neonates with CAs, the majority (65.5%) had not used folic acid supplementations during their indexed pregnancy, indicating their potential exposure to iron folate deficiency which in turn affected the normal development of the fetuses especially in the processes of neurulation.

Among mothers interviewed, 6.0%, 1.2%, and 11.2% had a history of alcohol drinking, cigarette smoking, and exposure to passive smoking during their early pregnancy, respectively. On the other hand, 2.0% were exposed to X – ray during pregnancy; 45.0% and 53.4% had maternal illness and they used antibiotics during early pregnancy, respectively. 5.2 % were exposed to pesticides/herbicides during their pregnancies.

22.7%, 19.0%, and 15.3% had used different types of unidentified drugs during the first three months, 4 -6 months, and the last three months, respectively. 92.4% had drunk coffee during their pregnancies. Besides, about 9.2% had a history of chewing khat during their pregnancy. 6.4%, 1.6% and 1.2% of the mothers had hypertension disorder, diabetes mellitus, and asthma, respectively. 2 (0.8%) of the participants used antiretroviral drugs during pregnancy.

Table 4:11: Mothers' response for selected risk factors for CAs

Exposures	Yes		No		Total number of respondantes
	Frequency	%	Frequency	%	
Folic acid use	89	35.5	162	64.5	251
Drink alcohol	15	6.0	234	94.0	249
Smoked cigarettes	3	1.2	248	98.8	251
Passive smokers	28	11.2	223	88.8	251
Exposure to X - ray	5	2.0	244	98.0	249
Exposure to pesticides	13	5.2	236	94.8	249
Diabetes mellitus	4	1.6	246	98.4	250
Maternal illness in early pregnancy	113	45.0	138	55.0	251
Have asthma	3	1.2	247	98.8	250
Drug usage during the first three months	57	22.7	194	77.3	251
Drug usage between the 4^{th} - 6^{th} months of pregnancy	47	19.0	201	81.0	248
Drug usage during the last three months of the pregnancy	38	15.3	210	84.7	248
Drink coffee during pregnancy	218	92.4	18	7.6	236
Use of khat during pregnancy	23	9.2	228	90.8	251
Hypertension disorder	16	6.4	234	93.6	250

Congenital Anomalies

A total of 290 different CAs were recorded from 251 cases of newborns. Of the 251 cases, 212 (84.5%) were single and the other 39 (15.5%) with multiple - two or more anomalies on a single case involving two or more organ systems. Most of the anomalies (93.5%) were major defects, while some were minor defects. Of the CAs identified, the proportion of NTDs, musculoskeletal defects including oro-facial clefts and abnormal limb development, gastrointestinal defects, urogenital defects, genetic disorders and CHD were 71.7%, 18.6%, 5.5%, 2.4%, 1.4% and 0.34%, respectively. Of 251 cases of newborns with CAs, 128 (51.0%) were males and 123 (49.0%) were females (Table 4:12).

About 35 (13.9%) of the victims of NTDs had associated with multiple anomalies. Of this, 22.8% of the NTDs were associated with other types of CAs other than types of NTDs and 77.1% were multiple NTDs cases. i.e. two or more NTDs observed on a single NTD case.

Table 4:12: Congenital anomalies by sex and organ systems in southwestern Ethiopia from 2016 to 2018

Birth defects	Male (n= 144)		Female (n = 145)		Total (n=290)	
	Frequency	%	Frequency	%	Frequency	%
NTDs	93	44.7	115	55.3	208	71.7
Musculoskeletal defects	35	64.8	19	35.2	54	18.6
Gastrointestinal defects	9	56.3	7	43.7	16	5.5
Urogenital defects	5	83.3.0	1	16.7	6+**1*** =7	2.4
CHD	0	0.0	1	100.0	1	0.35
Genetic disorders: Down syndrome, Achondroplasia	2	50.0	2	50.0	4	1.4

CHD: Congenital heart defect

1*: Ambiguous gentalia

Anencephaly, hydrocephalus and spina bifida were the most frequent CAs investigated with frequency of 72(24.8%), 58 (20.0%) and 55 (19.0%), respectively. The incidence rate of anencephaly, hydrocephalus and spina bifida were 20.5 per 10,000 births, 16.5per 10,000 births and 15.7 per 10,000 births, respectively. Whereas, ambiguous genitalia, CHD and duodenal atresia were the least frequent with incidence rate of 0.3 per 10,000 births each. The incidence of the different types of CAs are shown in Table 4:13.

Table 4:13: Frequency of CAs by types and their incidence rates per 10,000 births in southwestern Ethiopia (n = 251)

Types of CAs	Frequency	%	Incidence rate per 10,000 births
Anencephaly	72	24.8	20.5
Hydrocephalus	58	20.0	16.5
Spina bifida	55	19.0	15.7
Clubfoot-bilateral	20	6.9	5.7
Cleft lip	13	4.5	3.7
Limb defects (upper and lower limbs)	11	3.8	3.1
Meningomyelocele	9	3.1	2.6
Microcephaly	7	2.4	2.0
Encephalocele	7	2.4	2.0
Umbilical hernia	7	2.4	2.0
Cleft palate	7	2.4	2.0
Diaphragmatic hernia	3	1.0	0.86
Omphalocele	3	1.0	0.86
Imperforate anus	3	1.0	0.86
Gastroschisis	2	0.7	0.6
Hypospadias	2	0.7	0.6
Undescended testes	2	0.7	0.6
Down syndrome	2	0.7	0.6
Achondrplasia,	2	0.7	0.6
CHD	1	0.34	0.3
Ambiguous genitalia	1	0.34	0.3
Duodenal atresia	1	0.34	0.3
Eye defects	1	0.34	0.3
Renal agenesis	1	0.34	0.3

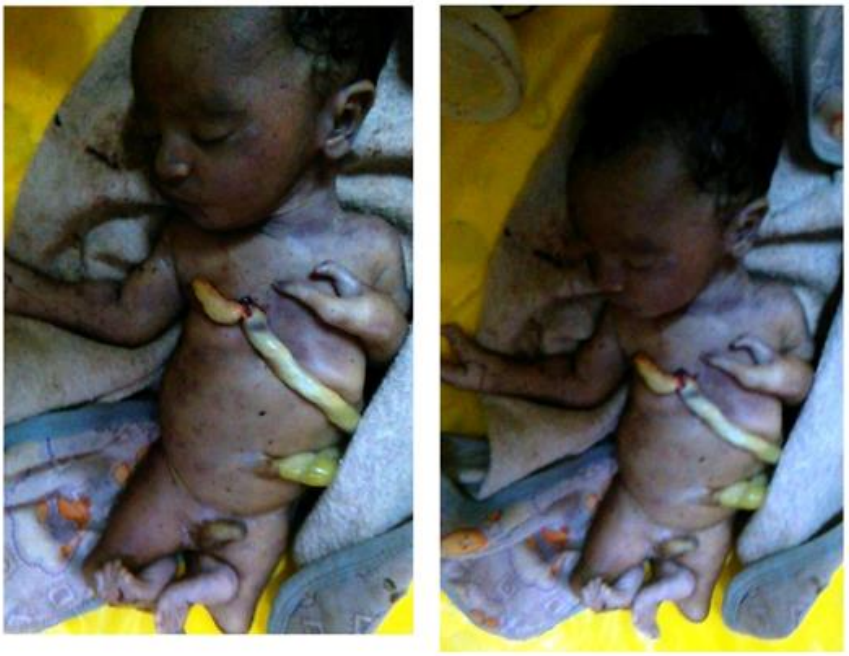
CHD, Congenital heart disease

a. Omphalocele with anencephally b. Hydrocephalus

Figure 4:3 Pictures showing omphalocel with anencephaly (a) and hydrocephalus (b), taken from Mettu Karl hospital during the study period.

(a) Omphalocele- the failurity of returning of physiologically herniated midgut loop during the 10^{th} week of prenatal life; Anencephaly – the failurity of the cranial neuropore to close at the cranial end of neural tube. (b) Hydrocephalus- over circulations and accumulations of cerebrospinal fluid in lateral ventricls of each cerebral hemisphere may be because of blockage of cerebral aqueduct of the midbrain.

Limb defects include: polydactyly, absence of arm and forearm, absence of fingers,presence of three digits and toe on both upper and lower limbs. As shown in figure



a. Upper and lower limb defects

b. Unilateral upper limb defects

Figure 4:4 Bilateral upper and lower limb defects (a) and unilateral upper limb defect (b) picture taken from JUSH (a) and Mettu Karl hospital(b) **during the study period** in southwestern Ethiopia**.** 7(a): bilateral hand defects were observed indicating that failurity of the palm and the phalangues to form which is commonly called aphalangia. Similary bilateral legs and feet defects were observed; figure 7 (b): unilateral right upper limb defects where the arm seems to

be developmentaly normal, where as the forarm and hand totally absent which is commonly called congenital upper limb amputations.

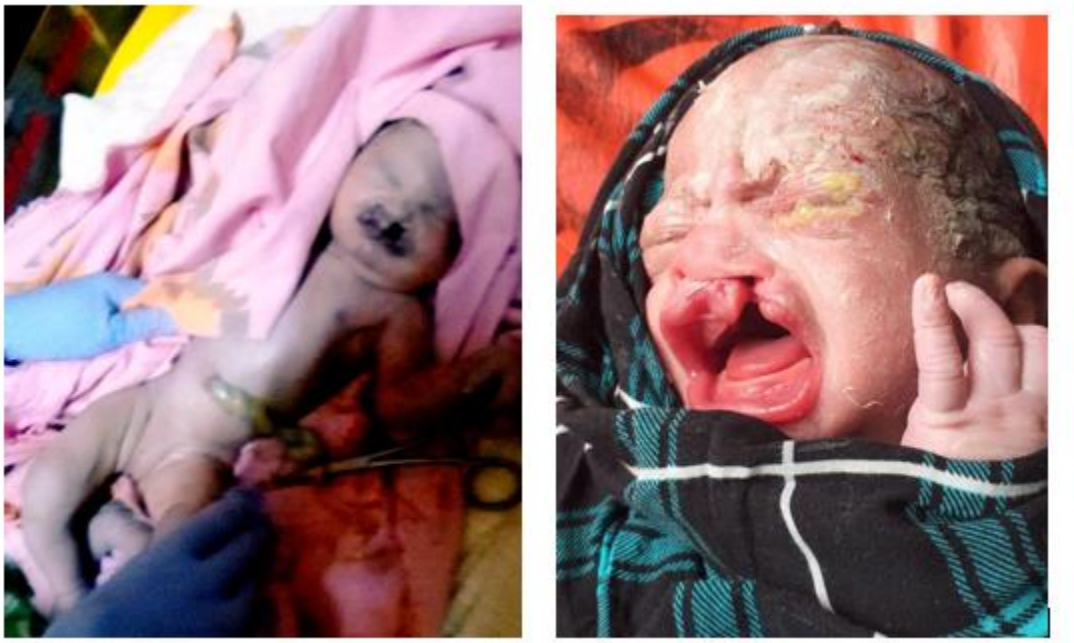
Sex based frequency distribution of major organ systems such as NTDs, musculoskeletal, gastrointestinal, and urogentiatl defects are shown in Table 4:14, 15, 16 and 17, respectively.

Table 4:144: Frequency distribution of NTDs by sex in southwestern Ethiopia

Neural tube defects	Male (n=93)		Female (n = 115)		Total (n= 208)	
	Frequency	%	Frequency	%	Frequency	%
Anencephaly	33	45.8	39	54.2	72	34.6
Hydrocephalus	26	44.8	32	55.2	58	27.9
Spina bifida	21	38.2	34	61.8	55	26.4
Meningomyelocele	5	55.6	4	44.4	9	4.3
Encenphocele	3	42.9	4	57.1	7	3.4
Microcephaly	5	71.4	2	28.6	7	3.4

Table 4:15: Frequency distribution of musculoskeletal defects by sex in southwestern Ethiopia

Musculoskeletal + Oro-facial defects	Male (n = 35)		Female (n= 18)		Total (n=53)	
	Frequency	%	Frequency	%	Frequency	%
Cleft lip	10	28.6	3	16.7	13	24.5
Cleft palate	4	11.4	3	16.7	7	13.2
Diaphragmatic hernia	2	5.7	1	5.5	3	5.7
Clubfoot	12	34.3	8	44.4	20	37.7
Limb defects (upper and lowe limb)	7	20	3	20	10	18.9



a. Bilateral cleft lip and palate b. Unilateral cleft lip and palate

Figure 4:5 Bilateral cleft lip and palate (a) and unilateral cleft lip and palate (b), photo taken at Nekemte referral hospital southwestern Ethiopia during the study period.

Table 4:16: Frequency distribution of gastrointestinal defects by sex in southwestern Ethiopia

Gastrointestinal defects	Male (n=9)		Female (n=7)		Total (n=16)	
	Frequency	%	Frequency	%	Frequency	%
Gastroschisis	1	11.1	1	14.3	2	12.5
Umblical hernia	4	44.4	3	42.9	7	43.75
Omphalocele	1	11.1	2	28.6	3	18.75
Duodenal atresia	0	0.0	1	14.3	1	6.25
Imperforate anus	3	33.3	0	0.0	3	18.75

Table 4:17: Frequency distribution of urogenital defects by sex in southwestern Ethiopia

Urogenital defects	Male (n=5)		Female (n=1)		Total(n=7)	
	Frequency	%	Frequency	%	Frequency	%
Renal agenesis	1	20	0		1	14.3
Hydronephrosis	0	0.0	1	100.0	1	14.3
Hypospadias	2	40.0	0	0.0	2	28.6
Undescended testes	2	40.0	0	0.0	2	28.6
Ambiguous genitalia	**		**		1	14.3

** Neither male nor female

4.3 Objective three: Risk factors associated with congenital anomalies

The sample consisted of 251 fetuses with CAs and 887 fetuses without CAs. Case to control ratio used were 1:4. Twenty four types of CAs were identified with total anomalies of 290 during the study period of 2016 to 2018. The number of cases and controls is shown in Figure 4:6. As indicated in Figure 9, 628 (55.2%) and 510 (44.8%) of the overall study participants were male and female fetuses respectively. 128 (51.0%), 122 (48.6%) and 1(0.4%) of the cases were male, female and ambigous genitalia, respectively. About 212 (84.5%) CAs identified were single/isolated, whereas 39 (15.5%) were multiple - two or more anomalies on a single case involving two or more organ-systems. The overall, frequency of CAs by organ system is shown in Table 4:18.

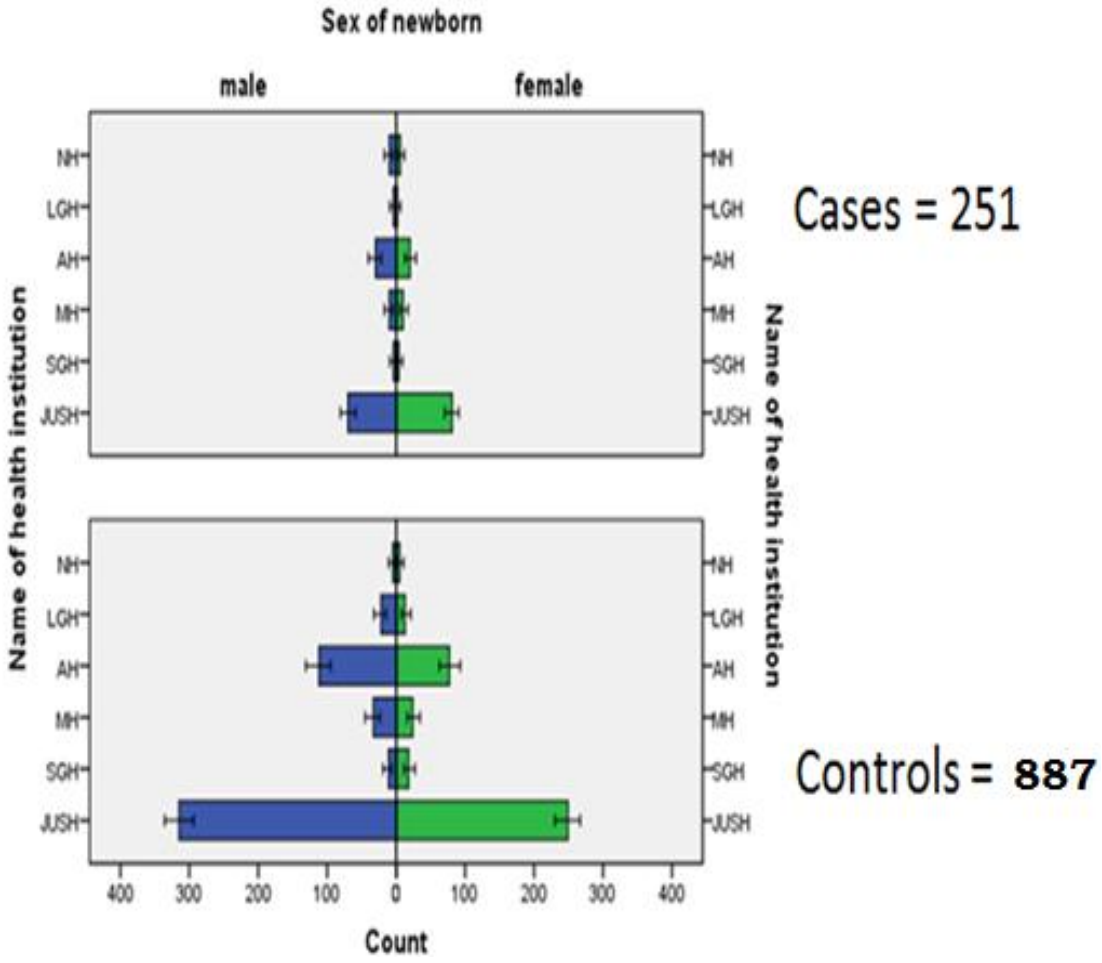


Figure 4:7 Distributions of the study participants by sex in selected hospitals in southwestern Ethiopia

AH: Agaro hospital; **JUSH:** Jimma University Specialized hospital; **LGH:** Limu Genet hospital; **MKH:** Mattu Karl hospital; **NH:** Nekemte hospital; **SGH:** Shanan Gibee hospital

Table 4:18: Frequency of CAs by sex, organ/organ systems among study subjects in southwestern Ethiopia (n=1138)

Types of congenital anomalies	Frequency	%
Neural tube defects	177	15.55
Musculoskeletal defects	46	4.04
Gastrointestinal defects	16	1.41
Urogenital defects	6+1* =7	0.62
CHD	1	0.09
Genetic disorders: Down syndrome, Achondroplasia	4	0.35
No congenital Anomalies/controls	887	77.94

CHD: Congenital heart defect; **1***: Ambiguous genitalia

Socio-demographic characteristics of the study participants.

The mean age for case and control mothers was 26 years and 25 years, respectively. The maternal age of the cases ranged 17 to 42 years. Similarly, the maternal age of controls ranged from 15 to 40 years. Likewise, paternal age range for cases and controls were 20 to 61 years and 19 to 66 years, respectively.

About 26.0% and 21.0% of mothers of the cases and control were below 20 years, respectively. Whereas 32.4% and 37.5% mothers of the cases and controls were in the age group of 21 to 24 years, respectively. Besides, 38.0% and 37.4% of the mothers of the cases and controls were in the age group of 26 to 35 years, respectively. Lastly, 3.6 % of both mothers of the cases and controls were great or equal to the age of 36 years (Table 4:19).

Among the mothers of the cases and controls, about 60.6% had formal education while 39.4% of the mothers were illiterates. 5% had primary education, 31.5% had junior level, 38.1% had

attained high school level and 25.5% joined higher education. In terms of religion inference, 61.6%, 23.8% and 14.6% were Muslims, Orthodox and Protestants, respectively.

71.8%, 6.7% and 1.4% had monthly income of one thousand to five thousand, six thousand to ten thousand and eleven and above thousands Ethiopian Birr, respectively. Among the mothers of both cases and controls, 59.9% were housewives, 11.8% were farmers, 18.9% were governmental employees, 7.6 were merchants and 1.7% were unemployed. The socio-demographic characteristics of the study participants of cases and controls are shown in Table 4:19.

Table 4:19: Socio-demographic characteristics of the study subjects with or without CAs in southwestern Ethiopian

Characteristics		Cases (n = 251)		Control (n = 887)		Total (n = 1138	
		Frequency	%	Frequency	%	Frequency	%
Age of the mothers	<=20	65	26.0	189	21.4	254	22.4
	21 - 25	81	32.4	331	37.5	412	36.4
	26 - 35	95	37.8	330	37.4	425	37.5
	>= 36	9	3.6	32	3.6	41	3.6
Maternal education	Illiterate	120	47.8	327	37.0	447	39.4
	Literate	131	52.2	556	63.0	687	60.6
Maternal educational level	1 -4 (primary)	13	9.9	21	3.8	34	5.0
	5 – 8(junior)	46	35.1	169	30.6	215	31.5
	9 – 12 (high school)	49	37.4	211	38.2	260	38.1
	13 and above (Higher institution)	23	17.6	151	27.4	174	25.5
Religion	Muslim	164	65.3	535	60.6	699	61.6
	Orthodox	48	19.1	222	25.1	270	23.8
	Protestant	39	15.5	126	14.3	165	14.6
Average monthly income	< 1000	37	26.4	89	18.3	126	20.1
	1000 -5000	95	67.9	355	72.9	450	71.8
	6000 -10000	6	4.3	36	7.4	42	6.7
	11000 and above	2	1.4	7	1.4	9	1.4
Maternal occupation	Housewife	151	64.8	503	58.6	654	59.9
	Farmer	34	14.6	95	11.1	129	11.8
	Employee	29	12.4	177	20.6	206	18.9
	Merchant	11	4.7	72	8.4	83	7.6
	Unemployed	8	3.4	11	1.3	19	1.7
Fathers' age	<=25	55	22.6	236	29.7	291	28.0
	26 - 34	104	42.8	327	41.1	431	41.5
	>=35	84	34.6	232	29.2	316	30.4

About 7.2% of the mothers of the cases and 2.9% mothers of controls had no antenatal care visits. Regarding the birth order of the cases, 37.2%, 21.1%, 14.2%, and 27.0% were the first, the second, the third, the fourth and above (4+) babies to their families, respectively. For those controls, about 43.6%, 24.8%, 13.0% and 18.5% were the first, second, third, fourth and above (4+) babies to their families, respectively.

Regarding gestational age, about 47.6% of cases and 19.9% of the controls were classified as preterm (<37 weeks). The differences between the cases and the controls were statistically significant with Odds ratio of 2.461; 95% CI: 1.956 – 3.094, P- value < 0.001, revealing that cases are more likely to have premature birth. Hence, premature births were associated with the presence of CA. In contrary, 40.8% of the cases and 58.1% of the controls attendned their full tem of gestational age (Table 4:20).

As shown in Table 4:20, 39.8% and 8.2% of the cases and controls were born with low birth weight (<2500 g) respectively. About 61.6% and 5.2% of the cases and controls were sill births respectively. There were significant differences between the cases and the controls revealing that the presence of CAs could affect the neonatal outcome.

Table 4:20: Reproductive history of mothers who gave birth to neonates with or without CAs in southwestern Ethiopia

Characteristics		Case (n = 251)		Control (n = 887)		Total (1138)	
		Frequency	%	Frequency	%	Frequency	%
Antenatal care follow up							
Not attend at all		18	7.2	26	2.9	44	3.9
1 to 3 visits		138	55.5	311	35.2	449	39.7
Minimum of 4 visits		94	37.6	546	62.0	640	56.5
Parity	0 & 1	134	54.5	537	61.7	671	60.1
	2 & 3	68	27.6	217	24.9	285	25.5
	>= 4	44	17.9	117	13.4	161	14.4
Gravid	0 & 1	90	36.4	377	43.4	467	41.8
	2 & 3	83	33.6	321	36.9	404	36.2
	>= 4	44	30.0	171	19.7	245	22.0
Onset of labor	Spontaneous	197	79.1	799	91.6	996	88.8
	Induced	52	20.9	73	8.4	125	11.2
Mode of delivery	Vaginal	208	83.2	629	71.5	837	74.1
	Scissoral	42	16.8	251	28.5	293	25.9
Gestational age	Preterm	119	47.6	176	19.9	295	26.0
	Term	102	40.8	514	58.1	616	54.3
	Post term	29	11.6	195	22.0	224	19.7
Sex of the newborn	Male	128	51.0	500	56.6	628	55.4
	Female	123	49.0	383	43.4	506	44.6
Birth weight in grams	<2500	100	39.8	72	8.2	172	15.2
	>=2500	151	60.2	811	91.8	962	84.8
Birth out come	Alive birth	96	38.4	836	94.8	932	82.3
	Still birth	154	61.6	46	5.2	200	17.7
Types of birth outcome	Single	236	94.8	836	96.3	1072	96.0
	Twin	13	5.2	32	3.7	45	4.0
Birth order	1st	92	37.2	380	43.6	472	42.2
	2nd	52	21.1	216	24.8	268	23.9
	3rd	35	14.2	113	13.0	148	13.9
	4th and greater	67	27.0	163	18.5	230	19.5

About 12.4% and 9.2% mothers of the cases and controls had a history of abortion. However, the difference between mothers of the cases and controls were not statistically significant**.** 9.3% and 7.9% mothers of the cases and controls had past history of still births, respectively**.** About 3.0% and 0.8% of the cases and control mothers had birth history of CAs, respectively indicating that there were statistically significant differences between the case and the control mothers (COR = 3.590; CI: 1.247 – 10. 334; *P* – Value = 0.011) (Table 4:21).

About 3.8% mothers of the cases and 1.7% of controls had history of congenital malformation in their families revealing that there were statistically significant differences between the case mothers and control mothers (COR = 2.672; CI: 1.211 – 5.895; P- value = 0.012).

Table 4:21: Obstetric history of the study participants in southwestern Ethiopia

Variable		**Cases**		**Controls**		**COR (95% CI)**	**P-value**
		Frequency	**%**	**Frequency**	**%**		
History of abortion	Yes	29	12.4	81	9.2	1.467 (0.944 – 2.257)	0.085
	No	205	87.6	799	90.8	1	
History of still birth	Yes	22	9.3	70	7.9	1.223 (0.752 – 1.990)	0.416
	No	215	90.7	811	92.1	1	
Pervious history of CAs	Yes	7	3.0	7	0.8	3.590 (1.247 – 10.334)	**0.011**
	No	230	97.0	877	99.2	1	
History of CAs in the family	Yes	9	3.8	15	1.7	2.672 (1.211 – 5.895)	**0.012**
	No	225	96.2	861	98.3		

CI: Confidence interval**; COR:** Crude Odds ratio**; Reference = 1**

Associated risk factors (predictors)

There were maternal illness in 113 (45.0%) mothers of the cases and 373 (42.2%) of controls. 15 (6.0%) mothers of the cases and 48 (5.4%) of the controls had history of taking alcohol during early pregnancy/first trimester of pregnancy. 3 (1.2%) mothers of the cases and 2 (0.2%) control mothers had history of smoking cigarettes. Besides, passive smokers were observed in 28 (11.2%) mothers of the cases and 28 (3.2%) controls. 5 (2.0%) mothers of the cases and 5 (0.6%) of the controls had history of exposure to radiation. Exposure to pesticides and use of different antibiotics during their early pregnancy/ the first three months were observed in 13 (5.2%) and 79 (53.4%) mothers of the cases respectively. Whereas, 12 (1.4%) and 261 (46.9%) of the controls had exposure to pesticides and used different antibiotics during their early pregnancy, respectively.

57 (22.7%) and 77 (8.7%) of mothers of the cases and mothers of the controls had experiences of using unidentified medicines and drugs during the first three months of their pregnancies, respectively. Similarly, 47 (19.0%) and 38 (15.3%) mothers of the cases and 144 (16.4%) and 172 (19.5%) of the controls had history of using drugs in the second and third trimester of their pregnancy respectively (Table 4:22). 162 (64.5%) mothers of the cases and 449 (50.8%) of controls did not use folic acid supplementation during the index pregnancy respectively. Likewise, diabetes mellitus was observed in 4 (1.6%) mothers of cases and 1(0.1%) in controls. Besides, 16 (6.4%) mothers of the cases and 52 (5.9%) of the controls had hypertension before and during pregnancy. Asthma was observed in 4 (1.6%) mothers of the cases and 15 (1.7%) in controls.

Variables which were expected to contribute in the formations of CAs were entered into the binary logistic regression analysis to identify crud risk estimate. Of these, smoking cigarettes during pregnancy (COR =1.112; 95% CI: 0.883 – 31.263, *P*- value = 0.042), passive smoking (COR = 3.834; 95% CI: 2.225 – 6.607, *P*-value =<0.001), exposure to radiation (X –ray) during the early pregnancy (COR = 3.578; 95% CI: 1.027 – 12.459, P- value = 0.033), exposure to pesticides (COR= 3.998; 95% CI: 1.801 – 8.878, P – value <0.001), diabetic mellitus (COR= 14.096; 95% CI: 1.583 -125, P- value = 0.002), use of unidentified medication and drugs in the first three months of pregnancy (COR = 3.076; 95% CI: 2.110 – 4.482, P – value < 0.001) were

associated with CAs in the crude Odds ratio analysis and may be responsible for the occurrences of CAs. Conversely, folic acid (COR= 0.568; 95% CI: 0.425 – 0.760, P – value < 0.001) was considered to be a protective effect against the occurrences or the development of CAs.

Table 4:22: Bivariate analysis – COR for environmental, exposure to different chemicals and maternal illness in southwestern Ethiopia

Variables		Cases		Control		COR	95% CI		P -value
		(n =251)	%	(n = 887)	%		Lower	Upper	
Folic acid use	Yes	89	35.5	434	49.2	**0.568**	**0.425**	**0.760**	**0.000**
	No	162	64.5	449	50.8	1			
Drunk alcohol	Yes	15	6.0	48	5.4	1.112	0.612	2.022	0.727
	No	234	94.0	833	94.6	1			
Smoked cigarettes	Yes	3	1.2	2	0.2	**5.253**	**0.883**	**31.263**	**0.042**
	No	248	98.8	870	99.8				
Passive smoking	Yes	28	11.2	28	3.2	**3.834**	**2.225**	**6.607**	**0.000**
	No	223	88.8	855	96.8				
Exposure to X - ray	Yes	5	2.0	5	0.6	**3.578**	**1.027**	**12.459**	**0.033**
	No	244	98.0	873	99.4	1			
Exposure to pesticides	Yes	13	5.2	12	1.4	**3.998**	**1.801**	**8.878**	**0.000**
	No	236	94.8	871	98.6	1			
Diabetes mellitus	Yes	4	1.6	1	0.1	**14.096**	**1.583**	**125**	**0.002**
	No	246	98.4	880	99.9	1			
Use of antibiotics	Yes	79	53.4	261	46.9	1.298	0.903	1.867	0.158
	No	69	46.6	296	53.1	1			
Drug usage during the first three months	Yes	57	22.7	77	8.7	**3.076**	**2.110**	**4.482**	**0.000**
	No	194	77.3	806	91.3	1			
Drug usage between 4^{th} and 6^{th} months	Yes	47	19.0	144	16.4	1.195	0.830	1.720	0.337
	No	201	81.0	736	83.6	1			
Drug usage during the last three months	Yes	38	15.3	172	19.5	0.746	0.508	1.095	0.133
	No	210	84.7	709	80.5	1			
Use of khat during pregnancy	Yes	23	9.2	54	6.1	1.549	0.930	2.578	0.090
	No	228	90.8	829	93.9	1			

CI: Confidence interval; **COR**: Crude Odds ratio; **Reference** = 1

The variables with P- value of 0.2 and below in the COR were entered into multivariable logistic regression model adjusted to observe exposure variables association with CAs. As a result, unidentified drug usage in the first three months of pregnancy (AOR = 3.435; 95% CI: 2.286 – 5.160, P – value < 0.001), exposure to pesticides (AOR = 3.926; 95% CI: 1.659 – 9.289, P – value = 0.002), passive smoking (AOR = 4.104; 95% CI: 2.277 – 7.397, P – value <0.001), history of CAs in the family (AOR = 3.741; 95% CI:1.238 – 11.307, P – value = 0.019), surface water as a source of drinking water (AOR = 2.073; 95% CI: 1.385 – 3.100, P – value <0.001) were significantly associated with the occurrence of CAs. On the other hand, iron folate / folic acid supplementation during the indexed pregnancy (AOR = 0.639; 95% CI: 0.416 – 0.980, P - value = 0.040) had a protective effect against the development of CAs (Table 4:23).

Table 4:23: Responses of study participants on possible risk factors for CAs: Multivariable analysis of AOR for associated risk factors of CAs, southwestern Ethiopia

Variables		Cases		Controls		AOR	95% CI		P - value
		Number	%	Number	%		Lower	Upper	
Drug use during the first three months of pregnancy	yes	57	22.7	77	8.7	**3.435**	**2.286**	**5.160**	**0.000**
	No	194	77.3	810	91.3	1			
Exposure to pesticides	yes	13	5.2	12	1.4	**3.926**	**1.659**	**9.289**	**0.002**
	No	236	94.8	874	98.6	1			
Passive smoking	yes	28	11.2	28	3.2	**4.104**	**2.277**	**7.397**	**0.000**
	No	223	88.8	859	96.8	1			
Folic acid use	yes	89	35.5	438	49.4	**0.639**	**0.416**	**0.980**	**0.040**
	No	162	64.5	449	50.6	1			
History of CAs	yes	7	2.8	7	0.8	**3.741**	**1.238**	**11.307**	**0.019**
	No	244	97.2	880	99.2	1			
Water source for drinking									
Pipe water		169	67.3	716	80.7	1			
Underground water		26	10.4	72	8.1	1.492	0.889	2.503	0.130
Surface water		56	22.3	99	11.2	**2.073**	**1.385**	**3.100**	**0.000**
Antenatal care follow up									
Not attended at all		18	7.2	26	2.9	**2.952**	**1.456**	**5.984**	**0.003**
1 to 3 visits		138	55.2	310	35.1	**2.121**	**1.538**	**2.926**	**0.000**
Minimum of 4 visits		94	37.6	548	62.0	1			

CI: Confidence interval; **AOR**: Adjusted Odds ratio; **Reference** = 1

4.4 Objective four: Prevalence of neural tube defects and associated factors

NTDs were the most frequent CAs investigated in the present study. Of 251 cases of CAs identified, 177 cases were NTDs with total of 208 (82.9%) different types (Table 4: 24).

Table 4:24: Frequency of NTDs in selected hospitals in southwestern Ethiopia

Study hospitals	Cases (n=177)		Control (n=708)		Total (n=885)	
	Frequency	%	Frequency	%	Frequency	%
Jimma Medical Center	117	66.1	457	64.6	574	64.9
Shanen Gibe hospital	7	4.0	29	4.1	36	4.1
Mettu Karl hospital	13	7.3	57	8.1	70	7.9
Agaro hospital	26	14.7	138	19.5	164	18.6
Limmu Genet hospital	3	1.7	16	2.3	19	2.1
Nekemte hospital	11	6.2	10	1.4	21	2.4

Of the NTDs identified, anencephaly shown Figure 11, was the most prevalent with frequency of 72 (34.6%). 58 (27.9%), 55 (26.4%), 9 (4.3%), 7 (3.4%) and 7 (3.4%) were hydrocephalus, spina bifida shown in Figure 10, meningomyelocele, encenphocele and microcephaly, respectively (Table 4:25).

Table 4:25: Frequency distribution of types of NTDs in southwestern Ethiopia

Neural tube defects	Frequency	%
Anencephaly	72	34.6
Hydrocephalus	58	27.9
Spina bifida	55	26.4
Meningomyelocele	9	4.3
Encephalocele	7	3.4
Microcephaly	7	3.4

Figure 4:8 Picture taken at JMC, during the study period, showing the entire rupture of the spinal cord along with a vertebral column of the thoracolumbar region.

Spina bifida can occur when the caudal neuropore fail to close and can exist in different form. The most sever type is Myelomeningocele, where the spinal canal remain open along several vertebrae exposing the spinal cord.

Figure 4:9 Anencephaly. Picture taken at JMC during the study period in southwestern Ethiopia.

Anancephaly is a NTD that occurs when the cranial end of the neural tube fails to close, usually between the 23rd and 26th day following conception.

Socio-demographic characteristics of the study participants

The majority of the cases (66.1%) and the controls (64.6%) were from JUSH. The mean age of the case and control mothers was 25 years old. The minimum and maximum age range for the case was 18 years and 42 years; the minimum and maximum age range for the control mothers was 16 and 40 years. 42.4% and 45.8% of case and control mothers were under 25years. 46.3% and 48.1% of the case and control mothers were in the age range of 25 – 34 years. 11.3% of the case mothers and 6.1% of the control mothers belonged to the age range greater than 34 years. In this age range, the case mothers were more frequent than the control mothers revealing that there was statistically significant difference between the cases and the controls.

Regarding paternal age of the case fathers and the control fathers, 16.6% of the case fathers and 83.4% control fathers were under 25 years. 22.7% and 77.3% of the case and control fathers were in the age range of 25 to 35 years; 25.0% of the case fathers and 75.0% control fathers were greater than 35 years age range.

50.3% of the case mothers and 36.3% of the control mothers were illiterate. This shows that there was statistical significance between the case mothers and control mothers (COR =1.775; 95% CI: 1.273 – 2.474, *P*-value = 0.001). 49.7% of the case mothers and 63.7% of the control mothers had formal education. Of these, 21.6% of the case mothers and 27.6% of the control mothers joined higher institution. The differences were statistically significant at (COR = 3.729; 95% CI: 1.380 – 10.075, P – 0.009). This indicated that those mothers who had newborns with NTDs were more frequent than those who had higher education in references to the first category (1 – 4 grade level) in binary logistic regression.

Regarding the religions of the study participants, 62.0%, 24.1% and 13.9% were Muslims, Orthodox and Protestants respectively. 29.8% and 13.2% of the case and control mothers had monthly income less than one thousand Ethiopian Birr respectively (COR = 0.729; 95% CI: 0.133 – 4.002). 63.8% and 76.4% of the case mothers and control mothers had monthly income between one thousand and five thousands Ethiopian Birr respectively (COR = 1.967; (95% CI: 0.373 – 10.376).

Besides, about 4.3% of the case mothers and 9.1% of the control mothers had monthly income of six thousand to ten thousands Ethiopian Birr (COR = 3.500; 95% CI: 0.504 – 24.328). Only 2.1% of case mothers and 1.3% of the control mothers earned monthly income of eleven thousands and above.

On the bases of binary logistic regression analysis using the last as reference category, there was no association between the monthly income and occurrences of CAs. 59.2%, 11.0%, 20.1%, 8.0 and 1.6% of the study participants were housewives, farmers, governmental employees, merchants and daily laborers or unemployed, respectively. The socio-demographic characteristics of the study participants are indicated in the Table 4:26.

Table4:26: Socio-demographic characteristics of the study participants in southwestern Ethiopia

Characteristics		Cases (n = 177)		Control (n = 708)		Total (n = 885)	
		Frequency	%	Frequency	%	Frequency	%
Age of the mothers	<25	75	42.4	324	45.8	399	45.1
	25 - 34	82	46.3	340	48.1	422	47.7
	≥35	20	11.3	43	6.1	63	7.1
Age of the fathers	<25	39	16.6	196	83.4	235	29.3
	25 - 35	92	22.7	314	77.3	406	50.7
	>35	40	25.0	120	75.0	160	20.0
	Missing	6	3.4	78	11.0	84	9.5
Maternal education	Illiterate	89	50.3	257	36.3	346	39.1
	Literate	88	49.7	451	63.7	539	60.9
Maternal educational level	1 -4 (primary)	8	9.1	14	3.1	22	4.1
	5 – 8 (junior)	32	36.4	122	27.2	154	28.7
	9 – 12 (high school)	29	33.0	189	42.1	218	40.6
	13 and above	19	21.6	124	27.6	143	26.6
Religion	Muslim	121	68.4	428	60.5	549	62.0
	Orthodox	30	16.9	183	25.8	213	24.1
	Protestant	26	14.7	97	13.7	123	13.9
Average monthly in -come	< 1000 birr	28	29.8	51	13.2	79	16.5
	1000 -5000 birr	60	63.8	295	76.4	355	74.0
	6000 -10000 birr	4	4.3	35	9.1	39	8.1
	11000 birr and above	2	2.1	5	1.3	7	1.5
Maternal occupation	Housewife	110	62.1	394	55.6	504	56.9
	Farmer	22	12.4	74	10.4	102	11.5
	Employee	22	12.4	157	22.2	179	20.2
	Merchant	9	5.1	75	10.6	84	9.5
	Unemployed	8	4.5	7	1.0	15	1.7

Reproductive history of the study participants

As shown in Table 4: 27, 8.5% of the case and 3.1% of the control mothers did not have any antenatal care during their pregnancies. The case mothers who did not have antenatal care were more than twice of those control mothers who had the privilege of enjoying the antenatal care. 60.8% and 34.6% of the case mothers and control mothers had atteneded antenatal care 1 to 3 times, respectively (COR: 1.451; 95% CI: 0.715 -2.944, P = 0.302). 31.5% of the case mothers and 62.2% control mothers had atteneded their antenatal care follow up at least four times (COR: 5.079; 95% CI: 2.457 – 10.502, P value < 0.001).

About 11.5% and 9.1% of case and control mothers had history of abortion (COR = 0.770; 95% CI: 0.452 – 1.311, P = 0.336). As a result, history of abortion has no association with occurrence of NTDs. 9.6% case mothers and 7.8% control mothers had history of still births with significance level of binary logistic regression (COR = 0.796; 95% CI: 0.450 – 1.409, P=0.434). There was no association of still birth with the occurrence of NTDs.

2.8% and 0.8% of case and control mothers had previous birth history of giving birth to neonates with NTDs. A history of giving birth to neonate with NTDs of case mothers were three times more than that of the control mothers indicating that there was significant difference between the case and control mothers (COR = 3.401; 95% CI: 1.026 – 11.275, P = 0.045). There were significant differences between case and control mothers in terms of previous history of CAs in their families with frequency of 5.2% and 1.1%, respectively (COR = 4.761; 95% CI: 1.809 – 12.526, P = 0.002). The case and controls were matching in hospital survice, antenatal care visits, gender.

Table4:27: Selective reproductive history of the case and control mothers in southwestern Ethiopia

Characteristics		Case (n = 177)		Control (n = 708)		Total (885)	
		Frequency	%	Frequency	%	Frequency	%
Antenatal care follow up	Not attended at all	15	8.5	22	3.1	37	4.2
	1 to 3 visits	107	60.8	245	34.6	352	39.9
	Minimum of 4 visits	55	31.5	439	62.2	494	56.0
Parity	0 & 1	96	54.5	450	64.2	546	62.3
	2 & 3	48	27.3	164	23.4	212	24.2
	>= 4	32	18.2	87	12.4	119	13.6
Gravid	0 & 1	58	32.8	307	43.9	365	41.6
	2 & 3	67	37.9	260	37.1	327	37.3
	>= 4	52	29.4	133	19.0	185	21.1
Onset of labor	Spontaneous	131	74.0	641	91.6	772	87.2
	Induced	46	26.0	59	8.4	104	11.9
Mode of delivery	Vaginal	154	87.0	510	72.2	664	75.0
	Scissoral	23	13.1	196	27.8	219	24.8
History of abortion	Yes	20	11.5	64	9.1	84	9.6
	No	154	88.5	794	90.9	794	90.4
	Missing	3	1.7	27	3.8	30	3.4
History of still birth	Yes	17	9.6	55	7.8	72	8.2
	No	160	90.4	650	92.2	810	91.8
Birth history with CAs	Yes	5	2.8	6	0.8	11	1.2
	No	172	97.2	702	99.2	874	98.8
History of CAs in the family	Yes	9	5.2	8	1.1	17	1.9
	No	164	94.8	694	98.9	858	98.1
	Missing	4	2.3	5	0.7	9	1.0

Selective characteristics of the newborns with or without NTDs

Of the total cases (n=177) born with NTDs during the study period, 92 (52.0%) were females and 85(48.0%) were males. Similarly, of the total controls (n= 708) born without NTDs, 387 (54.7%) were males and 321 (45.3%) were females. There was no difference between male cases and male controls. In contrary, the case females (52.0%) was slightly higher than the control females (45.3%). Sex of newborns neither males (COR = 1.305; 95% CI: 0.938 – 1.815, P=0.114) nor females (COR = 0.766; 95% CI: 0.551 – 1.066, P = 0.114) was not associated with the occurrence of NTDs.

96 (54.2%) and 108 (15.3%) case and control mothers had newborns with gestational age of preterm birth, respectively. Significant differences were observed between preterm birth of case newborns with NTDs and control newborns without NTDs. This indicated that the preterm delivery was significantly associated with the presence of NTDs (COR = 6.584; 95% CI: 4.584 – 9.435, P <0.001). i.e. fetuses with NTDs were born earlier than the-full tem fetuses without NTDs. 83 (46.9%) case newborns with NTDs and 53 (7.5%) control newborns without NTDs were underweight – birth weight less than 2500g. The difference was statistically significant. (COR = 10.912; 95% CI: 7.265 – 16.390, P < 0.001).

Regarding the birth outcome, 129 (73.4%) of the case newborns and 32 (4.5%) of the control newborns were still births. The difference was significant between the case and control groups in its crude risk estimate (COR: 16.194; 95% CI: 11.410 – 22.982, P-value <0.001). Regarding the types of birth outcome of the newborns, 9 (5.1%) and 25 (3.6%) case and control newborns were twins, respectively. 28.8%, 32.8%,23.7% and 14.7% of the case newborns were the first, second, third and fourth and above to their families, respectively. Similarly, 18.9%, 43.4%,25.4% and 12.3% of the control newborns were the first, second, third and fourth and above to their families, respectively (Table 4:28).

Table4:28: Selective characteristics of the newborn with or without NTDs in southwestern Ethiopia

Characteristics		**Cases (n=177)**		**Control (708)**		**Total (n = 885)**	
Sex of the newborns	Male	85	48.0	387	54.7	472	53.3
	Female	92	52.0	321	45.3	413	46.7
Gestational age	Preterm	96	54.2	108	15.3	204	32.1
	Term	81	45.8	600	84.7	681	76.9
Birth weight in gram	<2500	83	46.9	53	7.5	136	15.4
	>=2500	94	53.1	655	92.5	749	84.6
Birth outcome	Alive birth	47	26.7	675	95.5	722	81.8
	Still birth	130	73.4	32	4.5	161	18.2
Types of birth out come	Single	168	94.9	666	96.4	832	96.1
	Twin	9	5.1	25	3.6	34	3.9
Birth order	1st	51	28.8	134	18.9	185	20.9
	2nd	58	32.8	307	43.4	365	41.2
	3rd	42	23.7	180	25.4	222	25.1
	4th and greater	26	14.7	87	12.3	113	12.8

Risk factors associated with NTDs

Among the study participants, 5.1% and 5.5% of the case and control mothers consumed alcohol during their pregnancies. 1.1% of case and 0.1% of control mothers had smoked cigarettes during their pregnancies. Similarly, 12.2% of case mothers and 3.4% of the control mothers had history of passive smoking. The difference was found to be significant between the cases and the controls. 1.7% and 0.7% of case and control mothers were exposed to X – ray during their early pregnancies, respectively. However, the overall exposure was minimal; there was significant difference between the case and the control mothers.

Besides, 5.7% of the case mothers who had newborns with NTDs and 1.6% of the control mothers who had newborns without NTDs were exposed to pesticides. The difference between the cases and controls were bold. Furthermore, 1.7% and 0.1% of the case and control mothers had diabetes mellitus, respectively. Likewise, 6.8% and 4.8% of the case and control mothers had hypertension disorders, respectively. Similarly, 45.2% of the case mothers and 48.0% of the control mothers had maternal illness during their pregnancies. For this reason, 53.7% and 52.4% of case mothers and control mothers had used antibiotics during their illness, respectively. Moreover, 1.1% of case mothers and 2.0% of the control mothers had suffered from asthma.

25.4%, 20.1% and 14.3% of case mothers who had newborns with NTDs had used unidentified medicine and drugs during the first three months, second three months and the last three months, respectively. Likewise, 9.2%, 18.8% and 21.8% control mothers had used unidentified drugs during the first three months, second three months and the last three months of their pregnancy, respectively. About 92.7% and 88.7% of case and control mothers consumed coffee throughout their pregnancies. No significant differences were observed between the case and control groups with regard to coffee intake.

Moreover, 9.0% of the case mothers and 4.9% of the control mothers chew khat during their pregnancies. The number of case mothers who had been chewing khat was nearly twice of the control mothers. The difference was significant between the case and the control groups. Lastly, 67.2% of the case mothers and 55.4% of the control mothers were not used to folic acid

supplementations during their index pregnancies. Meanwhile, 32.8% and 44.6% of the case and control mothers had taken folic acid during their pregnancies.

Selected variables which were considered to be selected for the occurrence of NTDs were entered to binary logistic regression for analysis of Odds ratio (OR) for crude risk estimate. Accordingly, staying with cigarette smokers, passive smoking during pregnancy (COR = 4.045; 95% CI: 2.211 – 7.402, P – value <0.001), exposure to pesticides (COR = 3.835; 95% CI: 1.602 – 9.181, P – value = 0.003), diabetes mellitus (COR = 12.191; 95% CI: 1.260 – 117.814, P – value = 0.031), drug usage during the first three months (COR = 3.372; 95% CI: 2.208 – 5.152, P – value < 0.001), use of khat during pregnancy (COR = 1.440; 95% CI: 1.032 – 3.538, P – value = 0.039) were associated with the occurrence of NTDs. In contrary, folic acid (COR = 0.605; 95% CI: 0.427 – 0.855. P –value = 0.004) was considered to have protective effect against the occurrence or the development of NTDs. The results of COR analysis is shown in Table 4:29.

Table4:29: Bivariate analysis – COR for environmental, family history, exposure to different chemicals and maternal illness in southwestern Ethiopia

Variables		Cases		Control		COR	95% CI		P - Value
		(n = 177)	%	(n=708)	%		Lower	Upper	
Folic acid use	Yes	58	32.8	316	44.6	**0.605**	**0.427**	**0.855**	**0.004**
	No	119	67.2	392	55.4	1			
Drunk alcohol	Yes	9	5.1	39	5.5	0.922	0.438	1.940	0.830
	No	168	94.9	667	94.5	1			
Smoked cigarettes	Yes	2	1.1	1	0.1	8.139	0.734	90.274	0.088
	No	175	98.9	704	99.9	1			
Passive smoking	Yes	22	12.4	24	3.4	**4.045**	**2.211**	**7.402**	**0.000**
	No	155	87.6	684	96.6	1			
Exposure to X - ray	Yes	3	1.7	5	0.7	2.435	.576	10.288	0.226
	No	174	98.3	698	99.3	1			
Exposure to pesticides	Yes	10	5.7	11	1.6	**3.835**	**1.602**	**9.181**	**0.003**
	No	167	94.3	696	98.4	1			
Diabetes mellitus	Yes	3	1.7	1	0.1	**12.191**	**1.260**	**117.914**	**0.031**
	No	174	98.3	703	99.9	1			
Drug usage during the first three months	Yes	45	25.4	65	9.2	**3.372**	**2.208**	**5.152**	**0.000**
	No	132	74.6	643	90.8	1			
Drug usage between 4th and 6thmonths	Yes	35	20.1	132	18.8	1.087	0.717	1.649	0.693
	No	139	79.9	570	81.2	1			
Drug usage during the last three months	Yes	25	14.3	153	21.8	**0.599**	**0.378**	**0.949**	**0.029**
	No	152	85.7	550	78.2	1			
Use of khat during pregnancy	Yes	16	9.0	35	4.9	**1.911**	**1.032**	**3.538**	**0.039**
	No	161	91.0	673	95.1	1			

CI: Confidence interval; **COR**: Crude Odds ratio; **Reference = 1**

Those variables with their value ending up with 0.2 and below in the COR were entered into multivariable logistic regression model to observe variable association with the occurrence of NTDs. Consequently, passive smoking (AOR = 4.171; 95% CI: 2.272 – 7.681, P – value < 0.001) exposure to X –ray, (AOR = 4.190; 95% CI: 1.074 – 16.346, P – value = 0.039), exposure to pesticides (AOR = 3.823; 95% CI: 1.606 – 9.106, P – value = 0.002), diabetes mellitus (AOR = 16.381; 95% CI: 1.614 – 166.241, P – value = 0.018), use of drug during the first three months (AOR = 3.284; 95% CI: 2.181 – 4.944, P – value < 0.001) and history of NTDs in the families (AOR = 2.592; 95% CI: 1.083 – 6.203, P – value = 0.032) were significantly associated with the occurrence or the development of NTDs. However, use of folic acid during early pregnancy (AOR = 0.678; 95% CI: 0.488 – 0.943, P – value = 0.021) had protective effect against the development of NTDS. Results of multivariable logistic regression are shown in Table 4:30.

Table 4:30: Responses of study participants on possible risk factors for NTDs: Multivariable analysis of AOR for associated risk factors of NTDs, southwestern Ethiopia

Variables		Cases		Control		AOR	95% CI		P - Value
		(n = 177)	%	(n=708)	%		Lower	Upper	
Folic acid use	Yes	58	32.8	316	44.6	**0.678**	**0.488**	**0.943**	**0.021**
	No	119	67.2	392	55.4				
Passive smoking	Yes	22	12.4	24	3.4	**4.171**	**2.271**	**7.681**	**0.000**
	No	155	87.6	684	96.6				
Exposure to X - ray	Yes	3	1.7	5	0.7	**4.190**	**1.074**	**16.346**	**0.039**
	No	174	98.3	698	99.3	1			
Exposure to pesticides	Yes	10	5.7	11	1.6	**3.823**	**1.606**	**9.106**	**0.002**
	No	167	94.3	696	98.4	1			
Diabetes mellitus	Yes	3	1.7	1	0.1	**16.381**	**1.614**	**166.24**	**0.018**
	No	174	98.3	703	99.9	1			
Drug usage during the first three months	Yes	45	25.4	65	9.2	**3.284**	**2.181**	**4.944**	**0.000**
	No	132	74.6	643	90.8	1			
History of giving birth to NTDs	Yes	5	2.8	6	0.8	2.194	0.662	7.277	0.194
	No	172	97.2	702	99.2	1			
History of NTDs in the family	Yes	9	5.2	8	1.1	**2.592**	**1.083**	**6.203**	**0.032**
	No	168	94.8	694	98.9	1			

CI: Confidence interval ; **AOR**: Adjusted Odds ratio; **Reference = 1**

4.5 Objective five: Genetic risk factors among mothers of affected newborns with congenital anomalies

Fifty study subjects who gave birth to newborns with CAs at the study hospitals were evaluated for the genetic risk of birth defects by using MLPA methods with specific focus on targets that are associated with the genetics of various types of CAs. The MLPA probe mix: P070-subtelomeric-mix that has probes for the telomeric regions of the 46 autosomal and sex chromosomes was used to determine the micro-deletion/duplication in sub-telomeres region of each chromosome. Accordingly, the MLPA analyses were performed on DNA obtained from 50 blood samples collected from the study subjects. Discernible test results were obtained from 92% of the samples, whereas 8% ofthe samples results were excludedas the internal MLPA quality control fragments indicated an insufficient genomic DNA denaturation in these samples.

Based on the MLPA analysis, of 46 target probes in the sample used, seven of them showed deletions and/or micro-deletions in the sub-telomere region of the targeted chromosomes. This included probes that target the KIAA0226-22 gene located 3q29 in 2.17%, RECQL4-17 gene located on 8q24.3 in 2.17%, EHMT1-10 gene located on 9q34.3 in 2.17%, NDN-1 located on 15q11.2, in 10.87%, THOC1-21 gene located on 18p11.32 in 2.17%, CHP2A-3 gene located on 19q13.43 in 4.35% and VAMP7-8 gene located on Xq28 in 2.17% of the study population (Table 4:31).

On the other hand, some of the specific probes that target genes on the different chromosome locations showed duplications/micro duplications in their sub telomere regions of the suspected chromosomes. This included: CHL1-3 located on 3p26.3 in 2.17%, GNB2L1-2 located on 5q35.3 in 2.17%, IRF4-3 located on 6p35.3 in 2.17%, TBP-2 located on 6q27 in 2.17%, IGSF9B-20 gene located on 11q25 in 2.17%, PSPC1 gene located on 13q12.11 in 2.17%, TM2D3-3 gene located on 15q26.3 in 2.17%, and ARSA-1 gene located on 22q13.33 in 2.17% of the study population (Table 4:31).

Table 4:31: Ddeletions or micro-deletion and duplications in sub-telomeres region of the targeted genes of study populations in southwestern Ethiopia

Probe length	Genes	Chromoso-mal band	Probe target	Final ration (FR)		
				FR<.7	0.7≤FR≤1.3	FR>1.3
306	TNFRSF18-4*	01p36.33	01-001.129416	-	46 (100%)	-
132	SH3BP5L-3*	01q44	01-247.077648	-	46 (100%)	-
315	ACP1-5*	02p25.3	02-000.267098	-	46 (100%	-
139	ATG4B-7	2q37.3	02-242.247199	-	46 (100%)	-
323	CHL1-3	3P26.3	03-000.336381	-	45 (97.8%)	1 (2.17%)
145	KIAA0226-22	3q29	03-198.883877	1 (2.17%)	45 (97.8%)	-
329	PIGG-8	4p16.3	04-000.505735	-	46 (100%)	-
152	FRG1-1	4q35.2	04-191.099071	-	46 (100%)	-
337	CCDC127-3	5P15.33	05-000.258919	-	46 (100%)	-
160	GNB2L1-2	5q35.3	05-180.601812	-	46(1oo%)	-
346	IRF4-3	6p25.3	06-000.339901	-	45(97.8%)	1 (2.17%)
166	TBP-2	6q27	06-170.707891	-	45(97.8%)	1 (2.17%)
355	SUN1-5	7p22.3	07-000.844978	-	46 (100%)	-
172	VIPR2-2	7q36.3	07-158.627924	-	46 (100%)	-
362	FBXO25-8	8p23.3	08-000.398391	-	46 (100%)	-
179	RECQL4-17	8q24.3	08-145.708631	1 (2.17%)	45(97.8%)	-
370	DOCK8-24	9P24.3	09-000.376335	-	46 (100%)	-
186	EHMT1-10	9q34.3	09-139.776962	1 (2.17%)	45(97.8%)	-
379	ZMYND11-2	10P15.3	10-000.215989	-	46(100%)	-
193	ECHS1-8	10q26.3	10-135.026355	-	46(100%)	-
387	BET1L-3	11p15.5	11-000.195448	-	46 (100%)	-
202	IGSF9B-20	11q25	11-133.292680	-	45(97.8%)	1 (2.17%)
393	KDM5A-23	12p13.33	12-000.286996	-	46 (100%)	-

211	ZNF10-5	12q24.33	12-132.242375	-	46 (100%)	-
402	PSPC1-1	13q12.11	13-019.254548	-	46 (100%)	1 (2.17%)
218	CDC16-8	13q34	13-114.027450	-	46 (100%)	-
409	PARP2-16	14q11.2	14-019.895643	-	46 (100%)	-
226	MTA1-7	14q32.33	14-104.991619	-	46 (100%)	-
418	NDN-1	15q11.2	15-021.482490	5 (10.87%)	41 (89.13%)	-
233	TM2D3-3	15q26.3	15-100.007800	-	45 (97.8%)	1 (2.17%)
427	DECR2-9	16p13.3	16-000.402226	-	46 (100%)	-
241	GAS8-11	16q24.3	16-088.637622	-	46 (100%)	-
436	RPH3AL-2	17p13.3	17-000.183588	-	46 (100%)	-
250	SECTM1-4	17q25.3	17-077.874063	-	46 (100%)	-
444	THOC1-21	18p11.32	18-000.204731	1 (2.17%)	45 (97.8%)	-
258	CTDP1-9	18q23	18-075.575783	-	46 (100%)	-
451	PPAP2C-7	19p13.3	19-000.232437	-	46 (100%)	-
265	CHP2A-3	19q13.43	19-063.755470	2 (4. 34%)	43 (93.48%)	-
459	ZCCHC3-1	20p13	20-000.226979	-	46 (100%)	-
274	UCKL1-7	20q13.33	20-062.046371	-	46(100%)	-
466	HSPA13-2	21q11.2	21-014.675469	-	46 (100%)	-
281	S100B-2	21q22.3	21-046.846658	-	46 (100%)	-
479	IL17RA-4	22q11.1	22-015.959666	-	46 (100%)	-
290	ARSA-1	22q13.33	22-049.413270	-	45(97.8%)	1 (2.17%)
490	SHOX-5	XP22PAR	X-000.521733	-	46 (100%)	-
298	VAMP7-8	Xq28	X-154.825695	1 (2.17%)	45(97.8%)	-

FR< 0.7: Showed deletion; **0.7≤FR≤1.3**: Normal copy number; **FR>1.3**: Showed duplication

The following electrophorograms showed decreased final ration of the genomic comparative analyses showing: a. Control sample (X); b. normal unaffected sample; c. affected sample in specific loci showing decreasing peak height. The first four fragments are a Q-fragment: quality control fragments: Q-82 (DNA) controls the quality and concentration of the DNA in the sample.

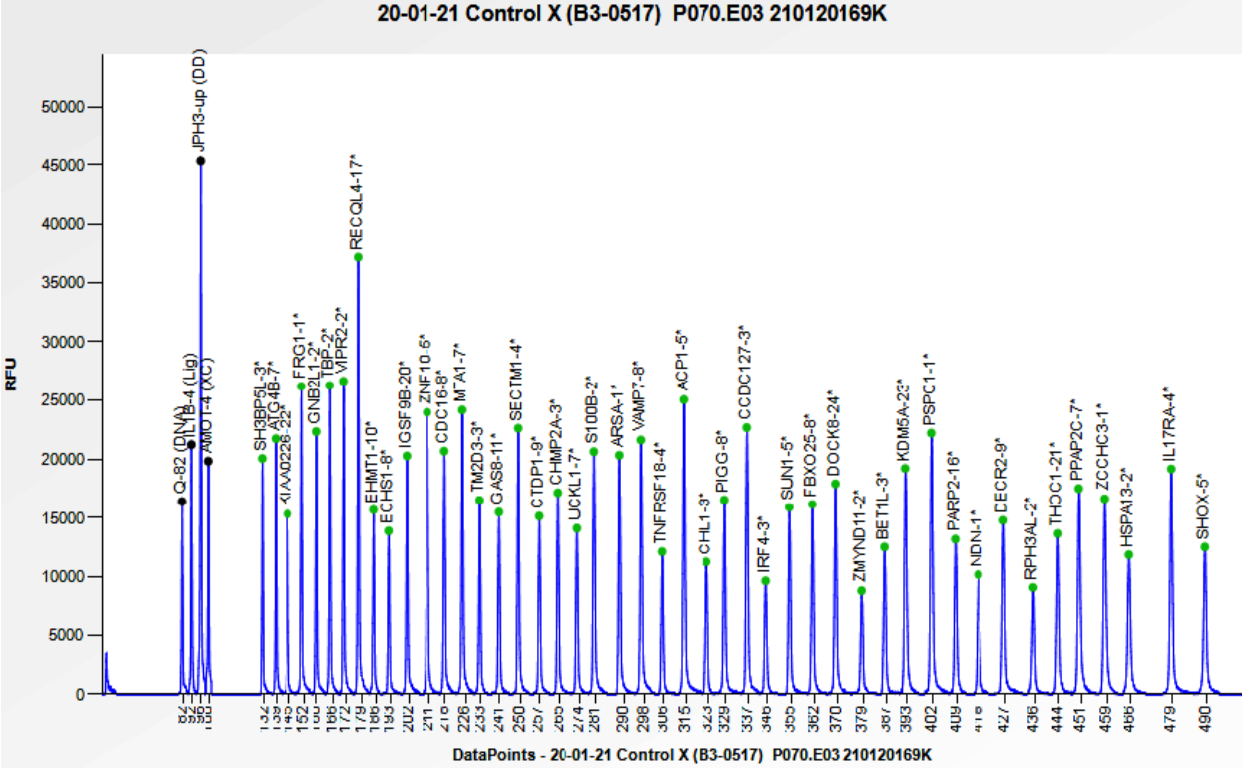


Figure 4:10 Electrophorogram of the control X chromosome showing signal ranges of the capillary electrophoresis in y – axis and the length on the X-axis.

MLPA fragment profile of a patient sample is at the bottom and that of the reference sample is on the top.

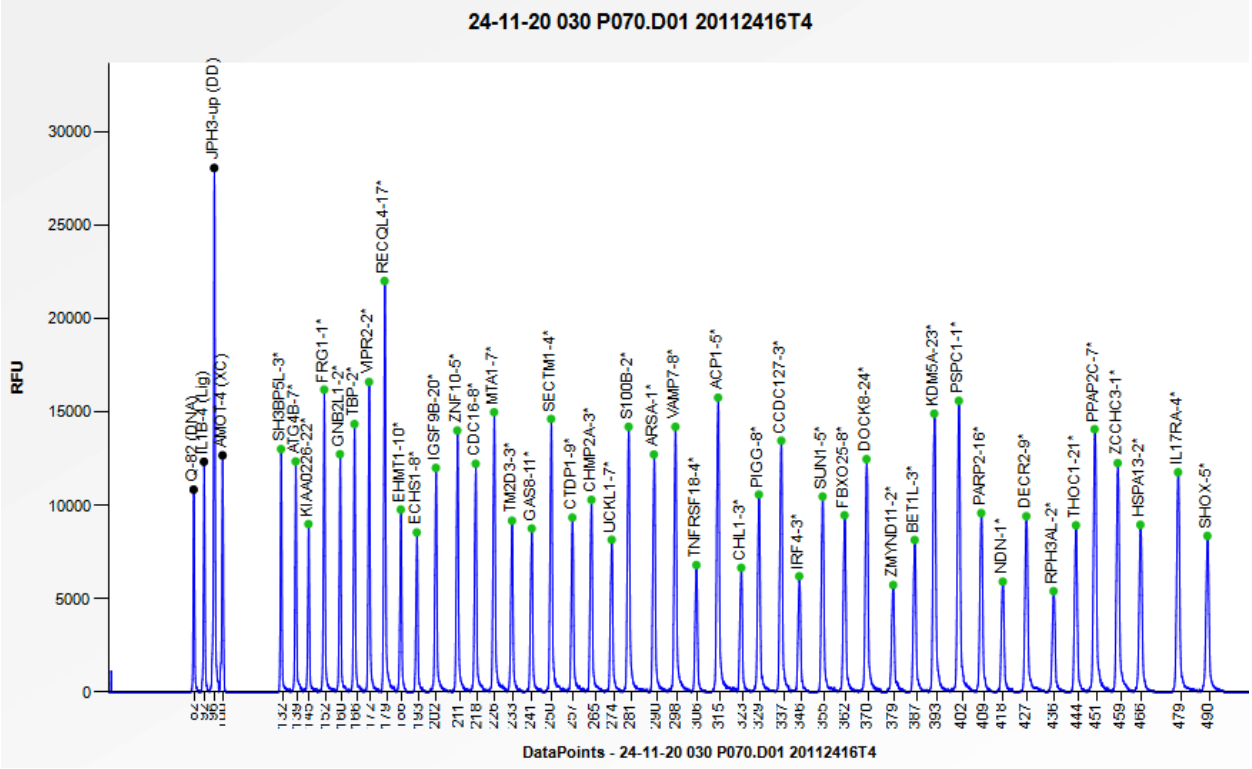


Figure 4:11 Electrophorogram study sample showing signal ranges of the capillary electrophoresis in y – axis and the length on the X-axis

MLPA fragment profile of a patient sample is at the bottom and that of the of a reference sample is on the top.

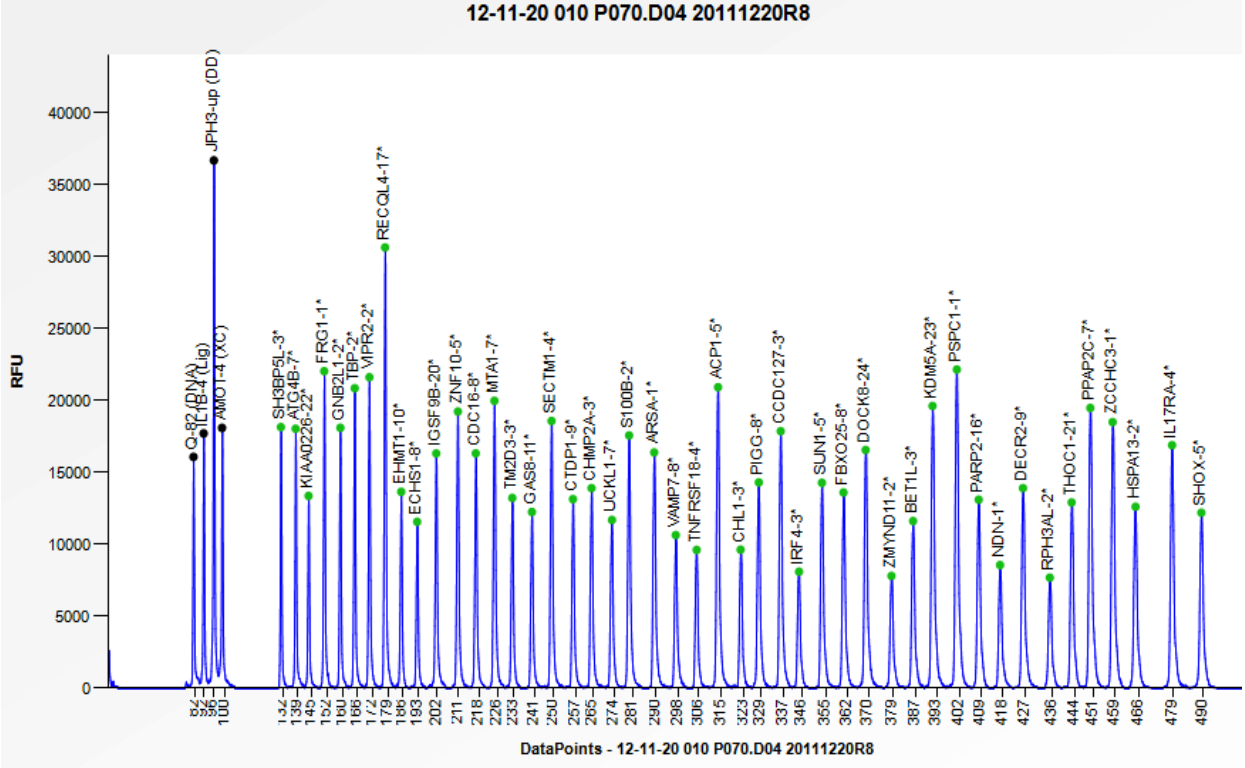


Figure 4:12 Electrophorogram showing signal ranges of the capillary electrophoresis in y – axis and the length on the X-axis.

MLPA fragment profile of a patient sample is at the bottom and that of the of a reference sample is on the top. Comparative analysis showed decrease in peak length of VAMP7-8 gene revealing deletion.

Chart ratio of the genomic comparative analysis

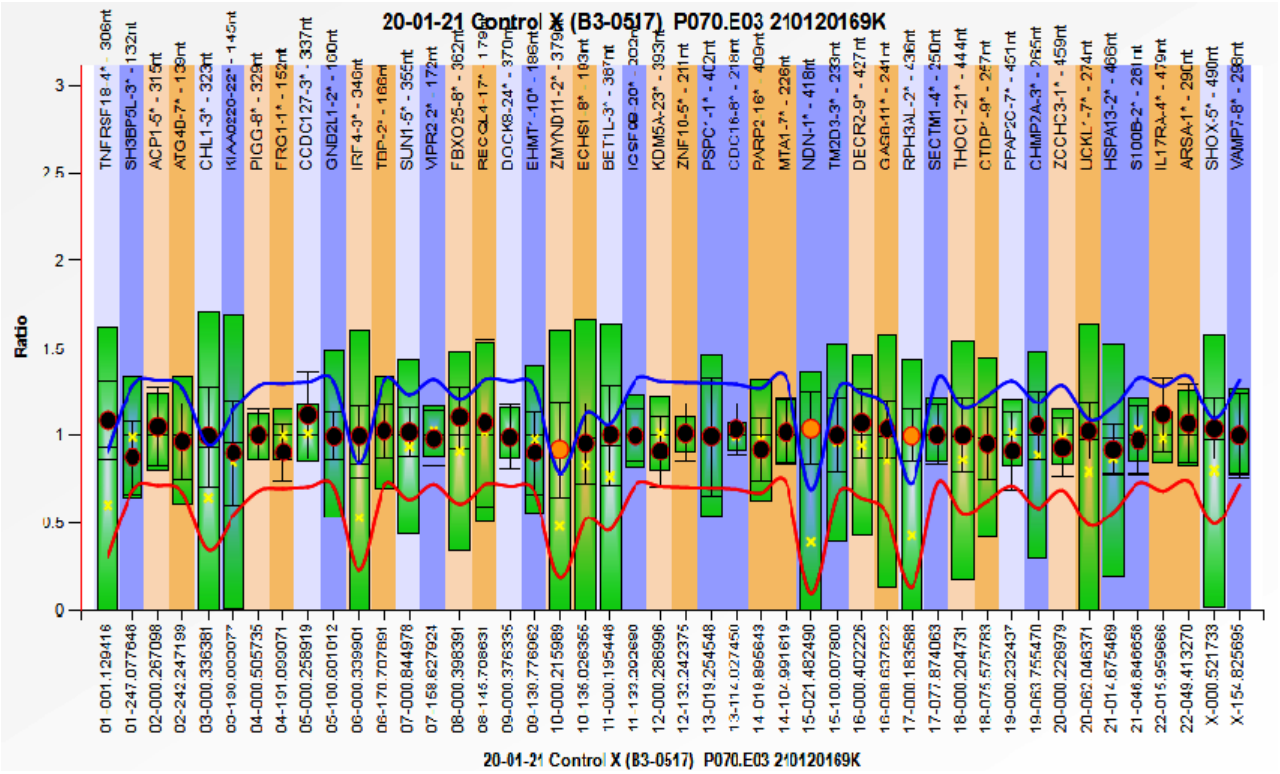


Figure 4:13 MLPA ratio chart showing control genome (X). A map view of all the locations of specific genome is displayed on the x-axis while the y-axis shows the final ratio of the probes.

The blue and red horizontal lines indicate the arbitrary borders showing normal final ration lying between 0.7 and 1.3. A final ratio less than 0.7 shows deletion whereas greater than 1.3 shows duplications. The black dots display final probe ratios and the error bar is with confidence interval of 95%. The yellow dots indicate the ambiguous comparative values.

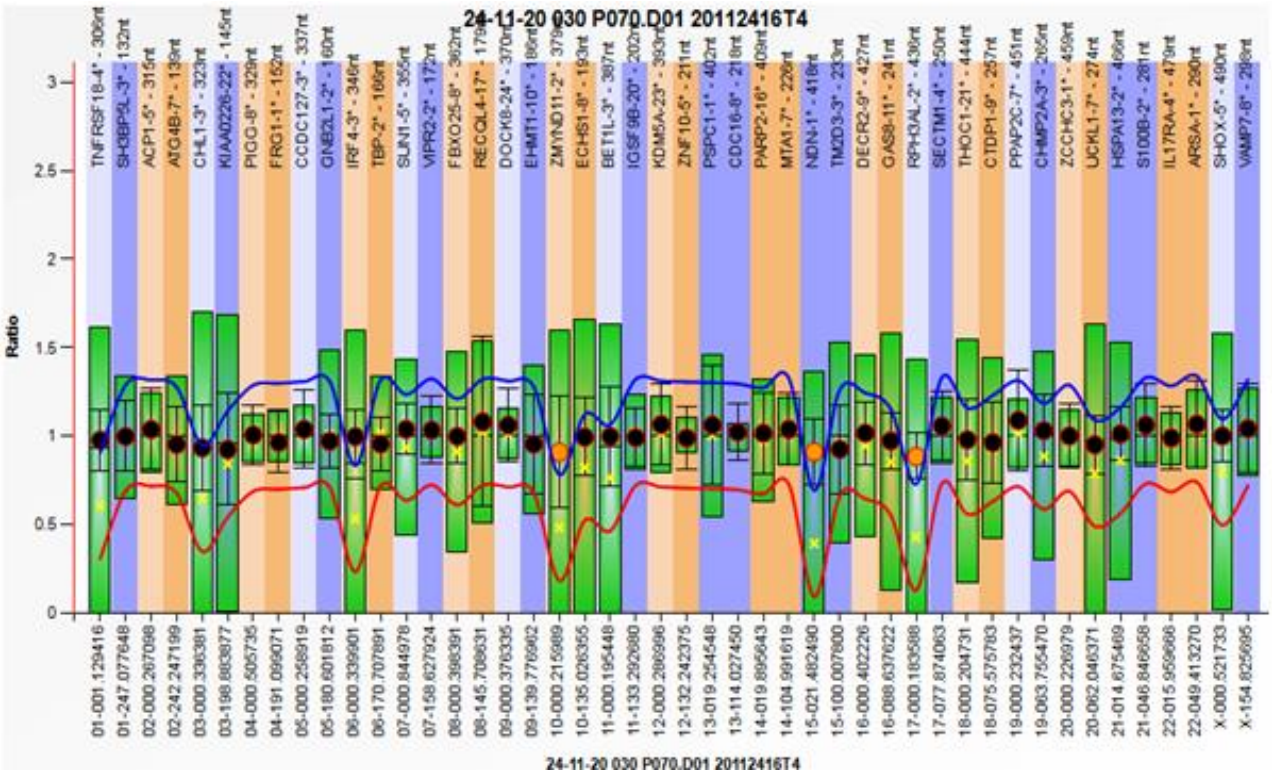


Figure 4:14 MLPA ratio chart showing unaffected (normal) DNA sample.

A map view of all the locations is displayed on the x-axis while the y-axis shows the final ratio of the probes. The blue and red horizontal lines indicate the arbitrary borders showing normal final ration lying between 0.7 and 1.3. The yellow dots indicate the ambiguous comparative values.

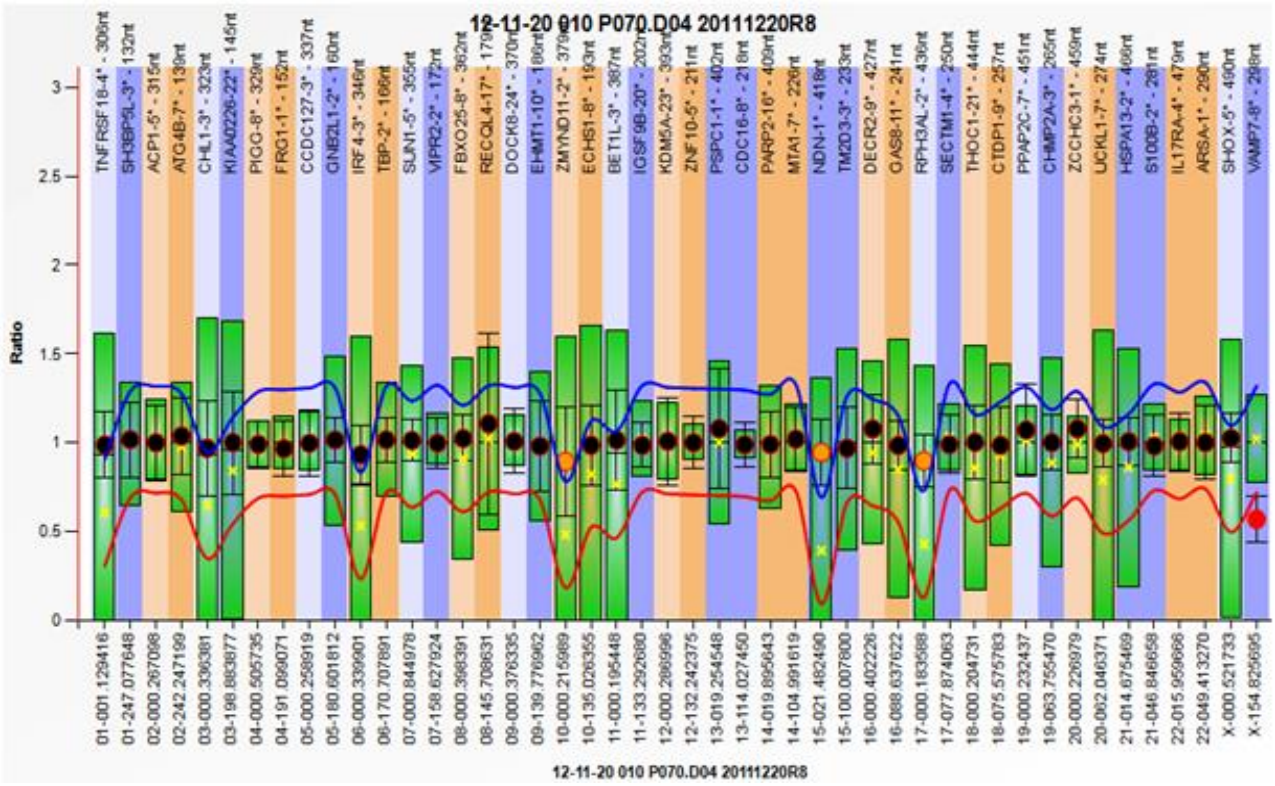


Figure 4:15 MLPA ratio chart showing specific deletion of VAMP7-8

The red dot display deletion of Xq28 with specific locus on VAMP7. Green box plots display regions associated with Xq28 (VAMP7) showing the final probe ratio of 0.57 which is less than 0.7. The yellow dots indicate the ambiguous comparative value.

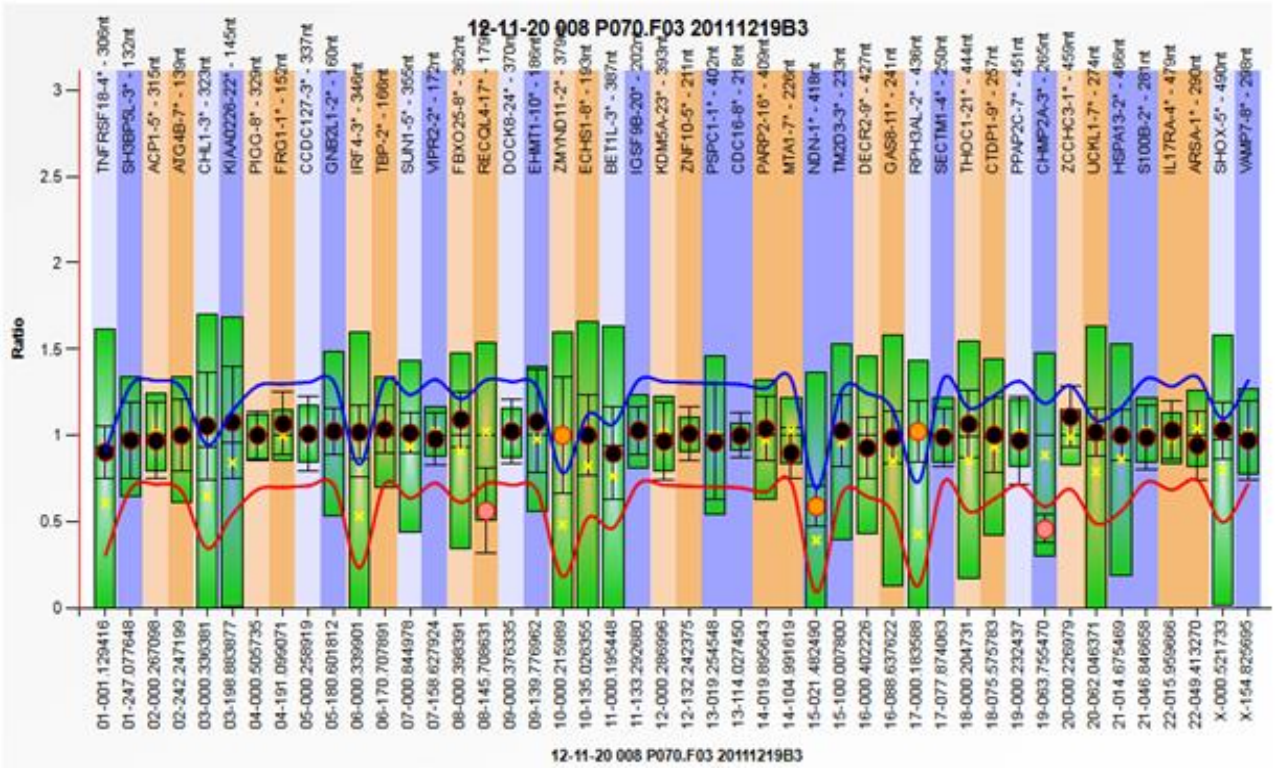


Figure 4:16 MLPA ratio chart showing specific deletion of RECQL4-17 AND CHP2A-3

The red dot display deletion of 8q24 and 19q13.43 with specific loci on RECQL4-17 and CHP2A-3, respectively. The final probes ratio of RECQL4-17 and CHP2A-3 are 0.56 and 0.46, respectively which is less than 0.7. The yellow dots indicate the ambiguous comparative value.

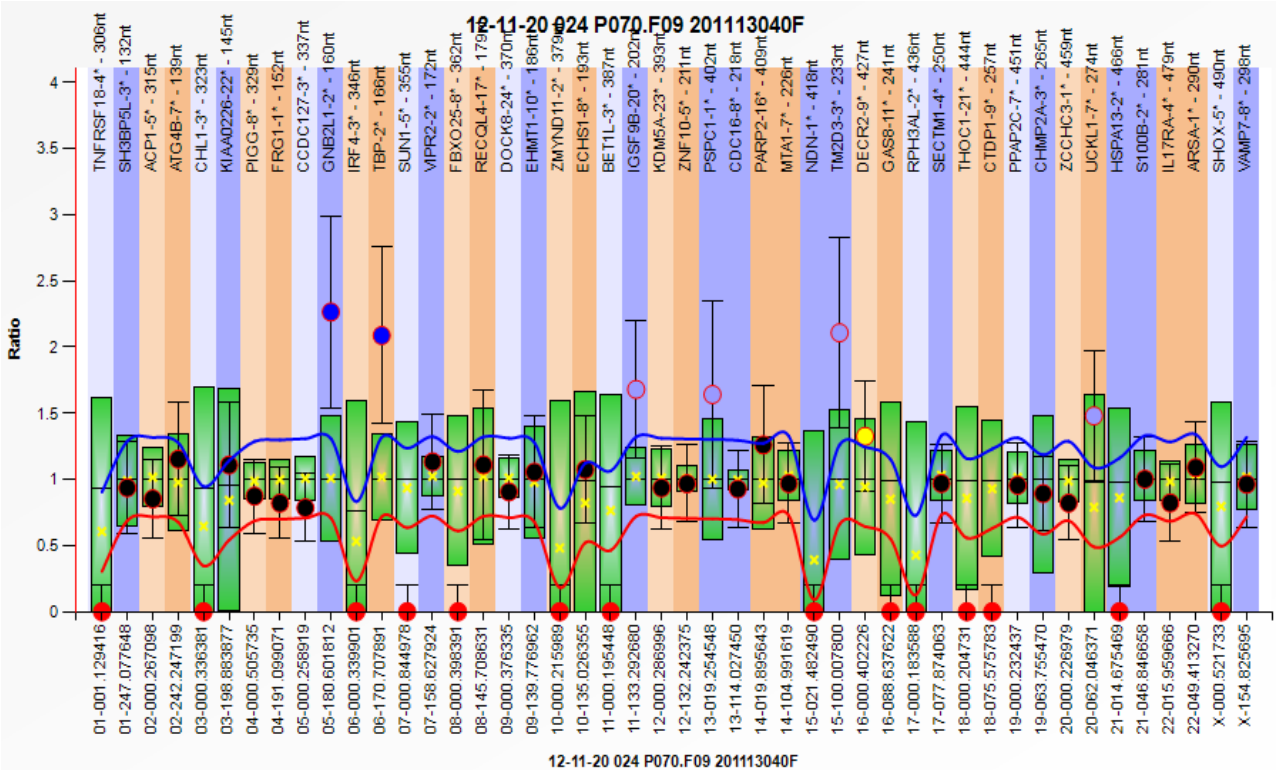


Figure 4:17 Ratio chart showing duplications of GNB2L1, TBP-2, IGSF9B-20, PSPC1, TM2D3-3 and ARSA-1 genes with final probe ratio of 2.27, 2.09, 1.68, 1.64, 2.11 and 1.48

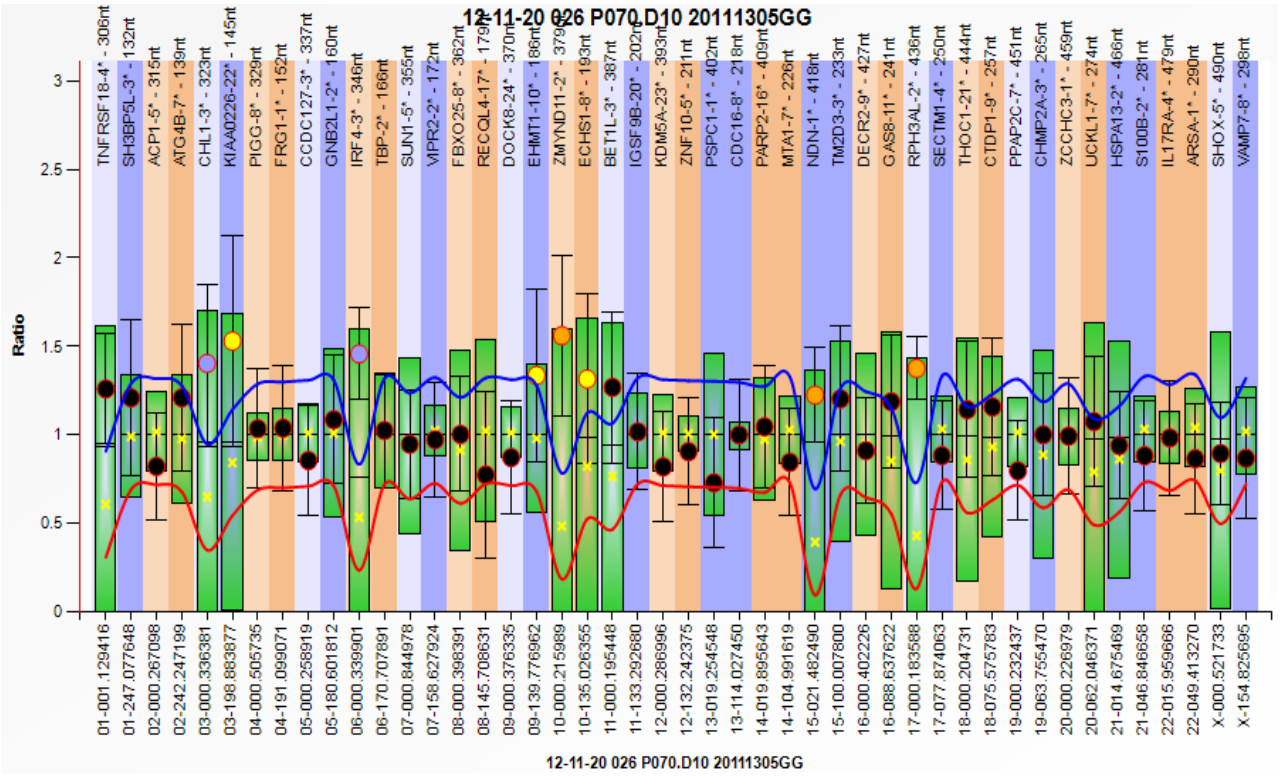


Figure 4:18 Ratio chart showing duplications of CHL1-3 and IRF4-3 genes with final ratio of 1.40 and 1.46.

Chapter Five

5. Discussion

Prevalence, types and patterns of birth defects

Birth defects, also called CAs, occur at a rate of 1 in every 33 newborn babies. Some of these CAs are minor and others are major defects. In most cases, the major defects are the leading causes of high perinatal mortality and morbidity in developing countries as well as in developed countries (Rosano et al., 2000).

According to World Health Statistics, about 260,000 neonatal deaths worldwide are caused by CAs accounting 7% of all neonatal deaths in the globe (Rizk et al., 2014). However, it is varying from 5% in south-east Asia to more than 25% in Europe. It was also indicated that between-country variations, from 4% (Bangladesh, Equatorial Guinea, Ethiopia, Liberia, Mali, and Sierra Leone) and an estimated 8% in China (Rizk et al., 2014).

In the present study, the overall proportion of CAs was 0.6%, whereas the study done in Addis Ababa and Amhara region showed a total proportion of 2 % (Taye et al., 2018). The difference might be because of socioeconomic differences between the two study populations within a different geographical locations or it might be because of the level of exposure to the causative agents.

In this findings, the overall prevalence rate of BDs was 5.5 per 1000 births, where NTDs were the most prevalent constituting 73.5% of all the defects. A report from the previous study done in referral hospital of northwestern Ethiopia showed that the NTDs were the most prevalent types of CAs with a frequency of 32.5% (Adane and Seyoum, 2018). However, the proportion in this findings was higher than that of northwestern Ethiopia. The difference might be because of the difference in a study setting where six hospitals were involved in this study while only one referral hospital was assed by Adane and Seyoum (2018) in the previous study; or it might be due to difference in the socioeconomic status of the study population.

In this study, the prevalence rate of NTDs was 40.05 per 10,000 births with predominant types of NTDs namely: anencephaly (13.7 per 10,000 births), hydrocephalus (13.2 per, 10,000 births), spina bifida (7.2 per 10,000 births), and meningomyelocele (3.9 per 10,000 births). The

prevalence rate of NTDs was twice (40.5 per 10000 births) when compared with the prevalence rate (20.1 per 10,000 births) of NTDs in China (Zhang et al., 2012). The prevalence rate of spina bifida (7.2 per 10,000 births) was slightly less than that of the prevalence rate of spina bifida (10.6 per 10,000 births) of the previous report in China (Zhang et al., 2012). This divergence might because of racial, geographical, environmental factors as well as genetic factors or multifactorial inheritances.

In this study, anencephaly and hydrocephalus were the most common types of CAs with a frequency of 25% and 24.6%, respectively. The remaining CAs made up 1.43 per 1000 births. In line with this findings, Abbey et al. (2017) reported that the prevalence of the major CAs at the University of Port Harcourt Teaching Hospital in the Niger Delta during the period 2011–2014 was 20.7 per 1,000 live births, with those of the central nervous system predominating at 27% of the total BDs. In both studies, the NTDs were more prevalent than the other CAs.

The study done in China identified that the prevalence rate of CAs was 15.6 per 1000 births (Zhang et al., 2012). In another study, the overall prevalence of the major CAs was 446.3 per 10,000 births in Korea (Jung-Keun et al., 2004). In both studies, the CAs seemed to be higher as compared to the present study. This might be because of differences in socio-demographic, socioeconomic status, racial or environmental factors.

Unlike the present study, where the NTDs were found to be the most prevalent defects, a study done in China indicated that heart septal defects (138.2 per 10,000) were the most prevalent followed by congenital hip dislocation (652 per 10,000) (Jung-Keun et al., 2004). The study done in Addis Ababa and Amhara region by Taye et al. (2018) revealed that oro-facial defects (34.2%) were the most prevalent followed by NTDs (30.8%). In contrast, NTDs (73.5%) were the most prevalent followed by a gastrointestinal defect (13.4%) in this study. This may be due to socio-economic, lifestyle, or demographic differences between the two study populations or might be because of the level of exposure to the causative agents.

Although,The male to female ratio in the present study was nearly 1:1 (51.8% and 48.2%), males are to some extent more affected than females. This maybe because of chromosomal abnormalities and gene mutations which commonly occur in both males and females under similar conditions. However, this needs further investigation to justify the condition. In contrast

to this study, there was a preponderance of females, with a female to male ratio of 1.4:1 as indicated in the study done in northwest Nigeria (Nnadi and Singh, 2017; Singh et al., 2015).

According to the European surveillance of CAs (EUROCAT) report, the prevalence of major CAs from 2003–2007 was 239 per 10,000 births, of which 80% were delivered, 17.6% were terminated by induced abortion, 2.5% died after birth, and 2% were stillbirths (Nnadi and Singh, 2017; Jung-Keun et al., 2004). This indicated that CA is the major cause of infant morbidity and mortality which is necessarly a major community burden.

The limitation of this part of the present study was that it was a hospital-based retrospective record review where some information was missing from the document. The outcome may not represent the actual prevalence of CAs in the southwestern Ethiopian population as it is logical to extrapolate every information might not be captured at each hospital.

Incidence of congenital anomalies at birth

The overall proportion of CAs observed in the present study was 0.72%. This finding is nearly similar to the result of a study conducted in the hospital of Fudan University in Shanghai, China, which showed CAs prevalence of 0.995% (Yang et al., 2017). Similar reports showed that the overall prevalence of CAs was 0.36% in China and 0.85% of the total newborns of the study done in Iran strongly support the results of the present findings (Sedighi et al., 2020; Ameen et al., 2018).

However, the prevalence of CAs observed in the present study was slightly less than the reported CAs from Addis Ababa and Amhara regions of Ethiopia with the prevalence of 1.96% and 1.61% (Adane and Seyoum, 2018; Taye et al., 2016). This difference might be due to the difference in geographical location, socioeconomic status, or level of exposure to risk factors like cigarrete smoking, exposure to passive smoking that may contribute to the occurrence of CAs. A similar study carried out in Brazil reported also the prevalence of CAs as 1.6% (Cosme et al., 2017).

The incidence of CAs observed in the current findings was 71.6 per 10,000 births and is slightly higher than the reported prevalence rate of the study conducted in Barbados with a prevalence rate of 62 per 10,000 (Singh et al., 2015). In contrary, the prevalence rate of the current findings

was significantly higher than the results of the study conducted in Lahore which reported the incidence of CAs to be 0.75 per 10,000 births (Parker et al., 2010) and less than the findings of the study carried out in Lebanon and Iran which showed incidence rates of 2.4% and 2.8% (Francine et al., 2014; Karbasi et al., 2009).

In this findings, the most common prevalent CAs were NTDs with a frequency of 71.7% followed by musculoskeletal defects with a frequency of 14.8%. The incidence rate of NTDs was 59.3 per 10,000 births, which is within the range of the reported global prevalence rate of 0.3 – 199.4 per 10,000 births (Zaganjor et al., 2016; Houcher et al., 2012; Abdolahi et al., 2014; Salbaum and Kappen, 2010; Pulikkunnel and Thomas, 2005).

Among the musculoskeletal defects observed, club foot (46.5%) is the most frequent, followed by cleft lip (30.2%), cleft palate (16.3%), and diaphragmatic hernia (7.0%). The most prevalent CAs observed were anencephaly with an incidence rate of 20.5 per 10,000 births (2.1/1000) followed by hydrocephalus with an incidence rate of 16.5 per10, 000 (1.7 /1000), while the least frequent were ambiguous genitalia, CHD, and duodenal atresia each with an incidence rate of 0.3 per 10,000 births (0.03/1000). The present findings were in agreement with the systematic review and meta-analysis done for 25 studies on a national estimate of birth prevalence of CAs in India (Bhide and Kar, 2018). According to Bhide and Kar (2018) anencephaly was the most frequent anomaly with a birth prevalence of 21.1 per 10,000 births in India.

Another systematic review made by Dewan et al. (2018) showed that hydrocephalus was the most prevalent in low and medium-income countries with an incidence rate of 123 per 100,000 births (12.3 per 10,000births) than the high-income countries with an incidence rate of 79 per 100,000 births (7.9 per 10,000 births). This systematic review strongly supports the result of the current finding in that hydrocephalus is the second most prevalent CAs with an incidence rate of 16.5 per 10,000 births.

The fourth most prevalent CA identified in the present study was club foot with an incidence rate of 0.6 per 1000 births (5.7 per 10,000 births). This is closely in agreement with the reported result from the study done in Sweden with an incidence rate of 0.93 per 1000 births (Danielsson, 1992). Moreover, the systematic review and meta-analysis of forty-eight studies over 20

countries for 55 years reported the incidence rate of club foot varied from 0.51 and 2.03 per 1000 births (Ahmed et al., 2017; Smythe et al., 2017) where the report of the present study is included within the range of the mata – analysis report of these 20 countries of low and medium-income countries revealing that Ethiopia itself is among the low-income countries.

The incidence rate observed in the present study was different from the prevalence rate of club foot reported by Kurdi et al.(2019), Parker et al (2009) and Ghirri et al. (2009) with the incidence rate of 1.29 per 1000 births. The discrepancy might be because of the scio-demographic and genetic factors as well as the level of exposure to risk factors associated to the development of clubfoot such as smoking ciggarates during early pregnancy, where the two countries populations had exposed to such risk factors differently.

The incidence rate of oro-facial cleft also varies in different populations. Accordingly, a high incidence rate was observed in Asians and American Indians with an prevalence rate of one in 5000 births. Likewise, slightly higher prevalence rate was observed in Africans with an occurrence rate of one in 2500 births. Furthermore, Parker et al. (2010) and Tanaka et al. (2012) reported the prevalence rate of orofacial cleft to be ranging from 7.75 to 10.63 per 10,000 births. In this findings, the oro-facial cleft: cleft lip with or without cleft palate had an incidence rate of 5.7 per 10,000 births.

However, the results of the present findings were not in the range of prevalence rate reported in the United States (7.75 to 10.63 per 10,000 births), It is in the range of WHO worldwide prevalence rate reported with the range varying from 3.4 to 22.9 per 10,000 births (WHO, 2001). This difference between the present findings and the reported prevalence rates in the USA might be because of the differences in the genetic makeup of the two study populations in different geographical locations, socioeconomic status, or level of exposure to risk factors such as, smoking in the first trimester of pregnancy and parity which were significantly associated with the occurrence of cleft lip revealing that maternal smoking in the present study participants were less experienced than those heavely smooking populations (Campos et al., 2016). The oro-facial cleft was the fourth most prevalent CA observed in the present findings.

Taye et al. (2016) and Adane and Seyoum (2018) reported in their retrospective, document reviewed studies conducted in Amhara regional state of Ethiopia that cleft lip is among the most common prevalent CAs next to NTDs. The difference between the result of the present study and the result reported in the previous studies done in central and northern Ethiopia may be due to the difference in study design and the duration of the study period or level of exposure to the causative agents for the occurance of cleft lip or might be due to socioeconomic status of the two geographical different populations of the same country. In the present study, the incidence rates of microcephaly, encephalocele, congenital umbilical hernia were 2.0 per 10,000 births each.

The prevalence rate of congenital umbilical hernia observed in this study was 2.0 per 10,000 births. This finding is by far lower from the prevalence rate of 15.85 per 1000 births reported in Turkish school children (Yücesan et al., 1993). The discrepancy might be because of the scio-demographic factors or racial differences between the Turkish and Ethiopian populations within different geographic locations or may be due to different genetic pool between the two populations or level of exposure to teratogens.

The prevalence of CHD of this study was in agreement with the prevalence rate of CHD reported from the research finding conducted in Shanghai, China with a frequency of 0.277%. Notwithstanding, the reported prevalence rate of hypospadias in Shanghai, China (0.11%) was less than the prevalence of hypospadias in this study of an incident rate of 0.6 per 10,000 births. Furthermore, the registry-based studies conducted in Europe indicated that the total prevalence rate of hypospadias was 18.61 per 10,000 births (Bergman et al., 2015).

Another CA observed in the present study was congenital diaphragmatic hernia (CDH) with a frequency of 3 (1.0%) and incidence rate of 0.86 per 10,000 births. The incidence rate of this study is much less than the incidence rate reported in Minnesota, USA with an incidence rate of 3.6 per 10,000 births in the USA. The incidence of CDH was 1.93/10 000 births, the overall CDH prevalence rate was 1.09 per 10 000 total births in southwest of Iran; 3.17 per 10,000 in Utah (Woodbury et al., 2019; Dehdashtian et al., 2017; Shanmugam et al., 2017; Kotecha et al., 2012). However, the result of the present study is included within the reported range of the incidence rate reported varying from 0.8 to 5 per 10,000 births.

The results of this findings showed that the incidence rate of omphalocele and gastroschisis was found to be 0.86 per 10,000 births and 0.6 per 10,000 births with a percentage of 1.0% and 0.7% respectively. The prevalence of omphalocele and gastroschisis reported from a retrospective study conducted in the Nigerian population was much lower than the present study with a prevalence of 0.16 % and 0.11% (Wokpeogu et al., 2018). Moreover, a study conducted in Sudan reported that the prevalence rate of omphalocele was 3 per100, 000 live births and that of gastroschisis was 0.3 in 100,000 live births (Ali and Ali, 2014) which is much less than the prevalence rate of the current study. On the contrary, the reported incidence rate of omphalocele was 3.38 per 10,000 births in France (Roux et al., 2018) and it was found to be much higher than the incidence rate of this findings.

Another type of CA investigated in the present study was imperforable anus, a congenital anorectal malformation where a normal anal opening is absent at birth (Levitt and Pena, 2007; Moore, 2006; Murphy et al., 2006). In this finding, the frequency of imperforable anus was found to be 1.0% of the total CA cases with an incidence rate of 0.86 per 10,000 births. The prevalence rate of imperforable anus in this study seems to be the least in Ethiopia as compared to the prevalence rates of 2.5 per 10,000 births in the USA, 1.4 per 10,000 births in Europe, and 5.7 per 10,000 births in South Africa (Thyen et al., 2006).

In the present investigation, ambiguous genitalia, CHD, and duodenal atresia were the least prevalent CAs with an incidence rate of 0.3 per 10,000 births each. But, there are no reported cases investigated before in the Ethiopian population. The epidemiological finding in Germany showed that the prevalence rate of ambiguous genitalia was 2 per 10,000 births (Thyen et al., 2006). The reported prevalence rate for isolated ambiguous genitalia was about 1 per 20,000 births in south America which is closely similar to the prevalence rate of the present study (Murphy et al., 2006).

Down syndrome and achondroplasia are genetic disorders observed in the present study with a prevalence rate of 1.14 per 10,000 births. The prevalence rate of Down syndrome in the present study was 0.6 per 10,000 births which is different from the prevalence rate in 10 regions of the United States which varried from 9.0 to 11.0 per 10,000 live births (Shin et al., 2009).

According to WHO, the predictable incidence of Down syndrom is between 1 in 1,000 to 1 in 1,100 live births all over the world (Al-Biltagi , 2015). The difference in prevalence among populations or countries or in the same population over time depend on the potential risk factors in common for that community. Likewise, achondroplasia is the most common of the skeletal dysplasias that result in marked short stature (dwarfism) with an incidence rate of 0.37 per 10,000 births (Coi et al.,2019; Mekonnen et al., 2020; Taye et al.,2019).

According to a population-based study conducted in the USA, the prevalence rate of achondroplasia varied from 0.36 per 10,000 births in New York to 0.6 per 10,000 births in Oklahoma (Pauli, 2019; Waller et al., 2018) strongly supporting the result of the present finding which showed that the prevalence rate of achondroplasia was 0.6 per 10,000 births. In this finding, more males were affected by musculoskeletal and gastrointestinal defects than females, 64.8% and 56.3% respectively.

Besides, reported sex distribution of CAs showed that more males were affected than females in Iraq 51.9% and 48.1%, in Nigeria 52% and 48%, and in China 58.7% and 40.7% (Yang et al., 2017; Abdolahi et al., 2014; Lisi et al., 2005).

Most research findings reported that advanced maternal age is strongly associated with higher risk of specific CAs (Goetzinger et al., 2016; Zhang et al., 2020). In the present study, maternal age greater than 36 years comprised 3.6% of the total CAs case mothers revealing that advanced maternal age was significanly associated with the occurrence of CAs. Several research findings revealed that maternal socioeconomic factors affect neonatal outcomes (Cantarutti et al, 2017; Chung et al., 2012; Mohamed and Aly, 2012; Waldan et al., 2007). Furthermore, compared to the uneducated or less educated mothers, the risk of having neonates with CAs are minimized in educated mothers (Cantarutti et al, 2017; Chung et al., 2012). In line with reported results from several studies, this finding revealed that 47.8% of mothers of the cases with CAs were illiterate mothers.

In the present study, increased risk of CAs was observed in primiparous (0 & 1) mothers with a frequency of 54.5%. Similar reports showed that women having their first birth had a significant association in having neonates affected with CAs as compared to the women having

their second birth and above (McNeese et al., 2015). On the other hand, some studies like Jawad et al. (2017) showed that the prevalence rate of CAs was higher among multiparous mothers as compared to the primiparous (Duong et al., 2012; Jawad et al., 2017; Malik et al., 2019). These studies demonstrated the association between increased parity and CMFs (Ekwochi et al., 2018). This is in contrast to a study that demonstrated more CMFs in primiparous mothers (Ameen et al., 2018; McNeese et al., 2015). The discrepancy may be elucidated by the fact that the effect of parity varies between different types of CAs.

In this findings, 12.4%, 9.3%, 3.0%, and 3.8% of the mothers who had given birth to neonates with CAs at birth had a history of abortion, stillbirths, previous birth history of CAs, and birth history of CAs in the family. Similar findings reported that the presence of CAs affected the neonatal outcome and were responsible for spontaneous abortions and stillbirths (Kokate and Bang, 2017; Kurinczuk et al., 2010). Moreover, the previous history in giving birth to a neonate with CAs and the history of CAs in the family may contribute to the occurrence of CAs in the next generation indicating that the genetic transmission can affect the next or the future newborns in a family.

In the present study, about 39.9% of the neonates born with CAs had low birth weight revealing that the presence of CAs can affect the birth weight of the neonates which may contribute to fetal growth retardation. The study done in northern Ethiopia indicated that parity, low birth weight, gestational age less than 35 weeks, male sex, and lack of antenatal care were significantly associated with CAs (Mekonen et al., 2015).

Furthermore, another study indicated that the prevalence rates of low birth weight and premature birth were significantly greater among infants with CAs than in their non-afflicted counterparts (Faal et al., 2018). In agreement with previous studies, the result of the present study indicated that 47.6% of the neonates with CAs were preterm (premature) births indicating the occurrence of CAs contribute to premature delivery with low births weight.

In the present study, maternal cigarette smoking, passive smoking and exposure to pesticides were observed in some mothers who had newborns with CAs. Some reported that they used to drink alcohol, chew khat, and take unidentified medicine or antibiotics during their early

pregnancies. Some experienced maternal illnesses during their earlier pregnancies and had exposure to radiation. Others reported that they had disorders such as diabetes mellitus, hypertension, and asthma. Similar studies reported that cigarette smoking, passive smoking, and exposure to pesticides were strongly associated with the occurrences of CAs (Alverson et al., 2011).

Moreover, using alcohol, unidentified medicine, chewing khat are significantly associated with the occurrence of CAs (Mekonnen, et al., 2020). Besides, the previous report showed that diabetes mellitus greatly contributed to the development of CAs (Correa et al., 2016). In the present findings, the uses of folic acid in most of the participants during early pregnancy were very low. This was in congruency with the study conducted in Addis Ababa and Amhara regions and northern Ethiopia in the Tigrai region (Taye et al., 2018; Berihu et al., 2019).

Despite the contribution of folic acid supplementation in preventing CAs, specifically NTDs, the low awareness of the mothers regarding the use of folic acid increase the occurrence of CAs across Ethiopia. Although, positive association of coffee consumption with the occurrence of CAs, especially NTDs was reported to be high (Schmidt et al., 2009), in the present study, about 92.4% of the mothers used to drink coffee throughout thier pregnancies. It is not evident that coffee consumption is a risk factor for the occurrence of CAs as this needs further investigation.

Associated risk factors with congenital anomalies

It was described that the human embryo is well protected in the uterus by an extra-embryonic membrane, although teratogens may cause developmental disruptions after maternal exposure to them in a specific period of organogenesis during a critical period in early pregnancy (Moore, 2011; Sunitha et al., 2002).

In this findings, maternal exposure to actual smoking in crud risk estimate, passive smoking during early pregnancy, and exposure to pesticides and herbicides during the critical period of embryogenesis had a significant association with the occurrence of CAs. Moreover, case mothers (1.2%) who used to smoke cigarettes during their pregnancies showed 6 times increased risk of getting neonates with CAs as compared to the control mothers (0.2%).

In line with the present study, in Iraq and Egypt maternal smoking either directly or passive in the first three months of pregnancy was strongly associated with the occurrence of BDs specifically cleft lip with or without cleft palate (Mohmmed et al., 2019). Conversely, a study done in southeastern Ethiopia, Bale zone, Oromia region by Mekonnen et al. (2020) reported that maternal exposure to pesticides during early pregnancy had a strong association with the occurrence of CAs with a frequency of 13.2% in exposed compared to 4.2% in unexposed subjects.

On the contrary, Taye et al (2018) reported that in Addis Ababa and Amhara region cigarette smoking either actual or passive smoking was not associated with CAs. However, this findings showed that there is an association of smoking with the occurrence of CAs. This is supported by several studies (Grewal et al., 2008; Alverson et al., 2011; Little et al., 2004; Hackshaw et al., 2011; Honein et al., 2000). The difference might be due to cultural differences in practicing cigarette smoking or staying with a smoker of a country with a geographically different location.

Likewise, Taye et al (2018) reported that maternal exposure to chemicals during early pregnancy had a significant association with the occurrence of CAs. Unlike reports from Addis Ababa and Amhara region where alcohol had a strong association with the risk of having a child with CAs, in the present findings, alcohol had no strong association with the occurrence of CAs. This difference might be due to the fact that in the present study most of the participants were Muslims and Protestants who are not used to comsume alcohol than in Addis Ababa and Amharic region.

In this findings, unidentified medicinal use during early pregnancy had a strong association with the occurrence of CAs. Some findings also reported that the use of unidentified medicine during early period of embryogenesis had a significant association with the occurrence of CAs (Taye et al., 2018; Adane and Seyoum, 2018; McNeese et al., 2015).

According to this findings, the use of surface water as a source of drinking water had a significant association with the occurrence of CAs. This might be due to the fact that chemicals and pesticides added to the surface water might have contaminated the surface water. Using contaminated water as a source of drinking water, especially during early pregnancy, may

contribute to the occurrence of CAs as it introduces chemicals that may cross the placenta and disrupt embryonic differentiation during the period of embryogenesis especially in the first 8 weeks of the embryonic period. The findings of the present study is in agreement with the finding of another study that reported that the maternal exposure to drinking water containing nitrate had a risk of having a baby with CAs (Croen et al., 2001).

Maternal diseases such as asthma and hypertension showed no association with the existence of CAs in the present study. However, maternal diabetes was significantly associated with the risk of having a fetus with CAs (McNeese et al., 2015). In contrast to the present findings, reports from several investigations showed that maternal diabetes has been well –established risk factor for CAs and adverse birth outcomes (McNeese et al., 2015; Reece, 2012; Croen et al., 2001). Besides, several findings described that gestational diabetes has a strong association with fetal growth abnormalities. Furthermore, pre-gestational diabetes was identified to be an important risk factor for structural anomalies as teratogenic effect of poorly controlled diabetes are considered to be the most important factors during the early period of pregnancy especial during the first 8 weeks at which time active differentiation of organ – system could occur (McNeese et al., 2015; Allen et al., 2007).

Correa (2016) reported that the embryopathy associated with pre-gestational diabetes mellitus is nonspecific underlying metabolic disorders. The same author further claimed that diabetes mellitus may increase the different signal transduction pathways and morphogenetic processes might be disturbed. Accordingly, maternal hyperglycemia resulted in increased glucose levels in the embryo, consequently, biochemical aberrations increase the oxidative stress that leads to cellular apoptosis (Salbaum and Kanppen, 2010).

Oxidative stress results in inhibition of the Pax3 gene especially in the processes of neurulation (Correa, 2016). Besides, oxidative stress occurred as a result of the imbalance between the production of oxygen free radicals and the antioxidant defense mechanism of the cells which can generate the irreversible oxidation of DNA leading to apoptosis as a result of enzymatic inactivation (Evers et al., 2009; Lary and Paulozzi, 2001).

The present study showed that there was a strong association between poor maternal folic acid supplementation during early pregnancy and the occurrence of CAs. Mothers who did get folic acid supplementation during their early pregnancies were strongly protected against having neonates with CAs, specifically NTDs. In other words, mothers who did not get folic acid supplementation during their early pregnancies were two folds more likely to have babies with CAs. Gebremichael and Welesamuel (2020) reported that folic acid supplementation in the early pregnancy reduces the occurrence of specific CAs.

In this study, mothers of the cases who did not get folic acid supplementation during early pregnancy was 64.5%, revealing that there is poor folic acid supplementation in southwestern Ethiopia. In agreement with the present findings, poor folic acid supplementation was observed in the Amhara region, northern Ethiopia (Adane and Seyoum, 2018). However, a study done in the Tigray region, northeastern Ethiopia, revealed that 40.9% of their study participants were adhering to iron folate supplementation (Gebremichael and Welesamuel, 2020).

As observed in this study, however, the Federal Ministry of Health and Regional Health Bureau promote folic acid supplementation to all women during their indexed pregnancy. Most of the pregnant women are not beneficiaries of such requirement yet. This might be because of poor awareness of antenatal care importances, or it might be due to resistance from the community cultural/ religious reseason.

According to the present study, 7.2% of mothers of the cases and 2.9% of control mothers attended no antenatal care follow up. The difference between the case and the control was statistically significant indicating that the mothers who did not receive ANC during their pregnancies were strongly associated with having babies with CAs. Similarly, mothers who had attended 1 to 3 ANC visits had a significant association with the presence of CAs compared to those who had ANC 4 visits. WHO (2016), recommends four minimum of ANC visits during pregnancy, where the 1st during early pregnancy, 2nd during the 28 -32 weeks, 3rd four weeks following the 2nd visits and the 4th ahead of the day of expected delivery. The pregnant mother can learn how to protect the pregnancy from certain causative agents during her visits. If not the case occurs without noticing the risk factors leading to CAs.

In this findings, maternal illness and the use of antibiotics during their gravidity showed a positive association with the occurrence of CAs. However, the association was not statistically significant. Conversely, Taye et al. (2018) found maternal illness and the use of antibiotics during early pregnancy has a strong association with the occurrence of CAs .

Although exposure to radiation especially, X –rays during early pregnancy seems to have an association with the occurrence of CAs in its crude risk estimate, in multivariable logistic regression it shows no association with the occurrence of the CAs.

The present study reported that mothers who used to drink coffee and to chew khat during their early pregnancies were not likely to have babies with CAs. Unlike the present study, the case-control study done in southeastern Ethiopia, Bale hospitals showed that chewing khat during the periconceptional period had increased risk of having neonates with CAs (Mekonnen et al., 2020).

In this finding, maternal history of abortion and stillbirth did not exhibit any association with the occurrence of CAs. However, the maternal previous birth history of CAs and the birth history of CAs in the family were slightly associated with the occurrence of CAs. This may have genetic implications. In this finding, more live births were observed in control than the cases. On the contrary, more stillbirths were observed in cases, newborns with CAs, (61.6%) as compared to the controls (5.2%). A similar report showed that the overall birth defect-specific stillbirth risk was increased among affected fetuses over the occurrence of stillbirths in general population in the United States (Dominique et al., 2020; Ehrhardt and Urquhart, 2014).

In the present study, about 39.9% of the newborns with CAs had low birth weight revealing that the presence of CAs can affect the birth weight of the neonates contributing to fetal growth restriction. The study done in northern Ethiopia also indicated that parity, low birth weight, gestational age less than 35 weeks, male sex, and lack of antenatal care were significantly associated with CAs (Mekonen et al., 2015; Walden et al., 2007).

Furthermore, another study indicated that the prevalence rates of low birth weight and premature births were significantly greater among infants with CAs. (Faal et al 2018). In

agreement with the previous studies, the result of the present study indicated that 47.6% of the neonates with CAs were preterm (premature) births and they were statistically significant.

In this study, socio-demographic characteristics such as maternal educational level, average monthly income, maternal occupation, and paternal age showed no significant association with the occurrence of CAs. Similarly, parity, gravidity onset of labor, mode of delivery, and types of birth outcomes and birth order of the newborns showed no significant associations with the occurrences of CAs.

Prevelence of NTDs and associated factors

CAs are the leading causes of infant mortality and morbidity throughout the world and the major causes of health problems in surviving children, especially in developing countries. Among several major CAs, NTDs are the common group of central nervous system anomalies affecting 0.5–2 per 1000 pregnancies worldwide (Mohmmed, 2019; Shawky and Sadik, 2011).

In the present study, 208 NTDs were identified from 177 NTDs cases constituting the most common types of CAs in the region. Among 290 different types of CAs identified, 208 (71%) were NTDs revealing a high incidence of NTDs in southwestern Ethiopia which is in agreement with Adane and Seyoum (2018) who reported that NTDs were the most frequent (32.5%) in the northeast Amhara region of Ethiopia.

The overall incidence rate of NTDs in this study was 50.5 per 10,000 births. The highest incidence of NTDs was observed in JMC with an incidence rate of 97.5 per 10,000 births. Similar findings were reported from retrospective studies done at three hospitals in Addis Ababa, central Ethiopia with a prevalence rate of 126 per 10,000 births (Gedefaw et al., 2018). Another hospital-based cross-sectional study done in the Tigray region reported that the incidence rate was 174 per 10,000 in eastern zone, 304 per 10,000 in southern zone and 72.8 per 10,000 births in western zone (Berihu et al., 2018). On the other hand, a retrospective study done in Amhara region, central and northern west Ethiopia reported that NTDs were the most prevalent with incidence rate 130 per 10,000 births (Taye et al., 2018).

Among the NTD cases identified, the incidence of anencephaly was 34.6% followed by hydrocephalus (27.9%) and spina bifida (26.4%). Encephalocele and microcephaly were the least frequent, 3.4% each. Similar reports from the studies in three hospitals in Addis Ababa reported that anencephaly was the most frequent (54.1%) followed by spina bifida (40.5%)(Gedefaw et al., 2018).

Unlike the present study where hydrocephalus was the second most frequent, report from three hospitals in Addis Ababa showed that spina bifida was the second most frequent NTD (Gedefaw et al., 2018). The discrepancy may be due to geographical, socio-demographic or socio-economic differences between the two populations and the level of exposure to risk factors, or may be due to different study settings or/and the duration of the study period. This is not a surprise for the prevalence and a type of CAs varies from country to country, even from region to region in the same country (Shawky and Sadik, 2018).

The present study showed that maternal age has no association with the risk of having a newborn with NTDs. However, maternal age above 35 years was considered to have an association with the risk of having a newborn with NTDs as there was difference between the cases (11.3%) and controls (6.1%). However, the difference was not statistically significant. This was found to be in line with reports from the study done in the Tigray region that showed maternal age above 35 years have a strong association with a high risk of having babies with NTDs (Berihu et al., 2019).

On the contrary, advanced paternal age is significantly associated with the occurrences of NTDs; a study done in Egypt reported the increasing paternal age above 50 years was associated with a high risk of NTDs (Yang et al., 20017; Shawky and Sadik, 2011). In the present study, paternal age had no association with the risk of having a child with NTDs. The difference might be because of socio-demographic factors or genetic factors or the maximum paternal age was 40 years old in the present study. The present finding was in agreement with the study done in the Tigray region in that no significant association was found between the age of the father and occurrences of NTDs (Berihu et al., 2019).

In current finding, maternal illiteracy was found to have a strong association with the risk of having a newborn with NTDs. On the contrary, a report from a study conducted in Tigray region, there was no significant association between maternal educational status and the occurrence of NTDs (Berihu et al., 2019). According to Berihu et al. (2019), the frequency analysis of most NTDs cases was classed below elementary school as compared to controls. The same is true in the present study.

In the present investigation, 8.0% and 3.1% of case mothers and control mothers did not attended antenatal care during their pregnancies. As there was a significant difference between the case and the control mothers who did not atteded the antenatal care revealed that antenatal care follow up was found to have a strong association with the risk of having a baby with NTDs. The same was reported from the study done in Egypt where 68.2% of the mothers did not receive antenatal care (Shawky and Sadik, 2011). Moreover, regular antenatal care may help early diagnosis and terminations of pregnancy incompatible with life (Afroze et al., 2020).

Unlike several scientific reports from different African countries including Ethiopia, the male sex was predominantly associated with the occurrence of CAs specifically NTDs because higher oxidative stress occurs more in the male embryo than in the female embryo (Dewangan et al., 2016; Sorri et al., 2015; Lary and Paulozzi, 2001; Evers et al., 2009). On the contrary, the present finding showed that females were relatively more affected by NTDs with a frequency of 52%. This is in line with the study done in Addis Ababa where females were slightly higher in proportion being affected with NTDs. (Gedefaw et al., 2018). Another study done in Iran reported that the sex of the newborn was not associated with NTDs (Talebian et al., 2015). On the other hand, Poletta et al. (2018) and Deak et al. (2008) reported that females (54.8%) were more affected with NTDs than males (45.2%).

In the present finding, passive smoking, exposure to radiation, and exposure to chemicals and pesticides during early pregnancy had significant association with the occurrence of NTDs. In agreement with the present findings, maternal obesity, inositol, chemical agents such as valproic acid, trichostatin and exposure to pesticides are among the causative agents of human NTDs (Van Gelder et al., 2010). Likewise, the use of unidentified medications during the first three months of pregnancy had a strong association with the occurrence of NTDs. The present study

also pointed out that the use of medication during early pregnancy was 3.3 times accountable for the occurrence of NTDs.

In this study, mothers with diabetes mellitus were 16 times prone to have a neonate with CAs specifically NTDs as compared to the control. This revealed that diabetes mellitus had a strong association with the occurrence of NTDs. In line with the present study, maternal diabetes significantly increased the risk of CMs during the first 8 weeks of intrauterine life in the human embryo (Xu et al., 2013; Pani et al., 2002). This is possible through elevated glucose concentrations that lead to oxidative stress and embryonic depletion of inositol (a simple carbohydrate that occurs in animal tissue and is a vitamin of the B group) leading to abnormal closure of the developing neural tube (Xu et al., 2013). Moreover, maternal diabetes induces autophagy impairment causing impaired neural tube formation by disrupting cellular homeostasis leading to endoplasmic reticulum stress and cellular apoptosis (Berihu et al., 2018; Gedefaw et al., 2018; Taye et al., 2018; Yang et al., 2017; Xu et al., 2013; Pani et al., 2002).

In the present findings, maternal folic acid supplementation during early pregnancy had a protective effect against the occurrence of NTDs. Several reports showed that maternal nutritional factors seem to contribute substantially to the complex etiologies of NTDs (Berihu et al., 2018; Gedefaw et al., 2018; Taye et al., 2018; Flores et al., 2014). Foremost among these factors is the periconceptional use of supplements containing folic acid, which is associated with a reduction in the risk of women having NTD-affected pregnancies (Ryan-Harshman and Aldoori, 2008). Folate metabolism is primarily related to the risk of NTDs in its disturbed pathways (Dunlevy et al., 2007). The current finding pointed out that poor folic acid supplementation was statically significant.

In the present findings, the use of khat during pregnancy seems to have a strong association with the occurrence of NTDs in its crud risk estimate in crud Odds analysis, the multivariable logistic regression in adjusted risk estimate in adjusted Odds analysis; it showed no association with the occurrence of NTDs.

Unlike a report from a study done in Addis Ababa and northern Ethiopia which showed that alcohol consumption had a strong association with the occurrence of CAs, the present study

showned no association with the occurrence of the NTDs. The discrepancy may be due to cultural differences between the Addis Ababa and Amhara regions where the subjects are used to drinking alcohol. In southwestern Ethiopia, most of the study participants were Muslims and Protestants who are not used to drinking alcohol; hence alcohol has no association with the occurrence of NTDS. Asthma, hypertension, and coffee drinking during pregnancies did not show association with the occurrence of NTDs.

Genetic risk factors for congenital anomalies

Detection of chromosomal abnormalities in sub telomere regions.

To the best of my knowledge, this study is the first to report the detection and duplication on the chromosomal bands among Ethiopian populations.

In the present study, genomic deletions, duplications and mutations in sub telomere regions of the targeted chromosomes using MLPA technique was detected. Sub telomeres are gene rich regions and these chromosomal deletions occur in these segments accounting for roughly 2.5% (Tashiro et al., 2017; Davidsson et al., 2008). In this study, the MLPA analysis confirmed the presence of deletion in the chromosome bands of 3q29, 8q24.3, 9q34.4, 15q11.2, 15q26.3, 18p11.32, 19q13.43, Xq28 from 50 sample population tested for chromosomal abnormalities.

The deletion of 3q29 is allied with neurodevelopmental and psychiatric phenotypes, as well as high risk for autism spectrum disorder (ASD). However, the phenotypic spectrum of the deletion, particularly with respect to ASD, remains poorly described (Pollak et a., 2019).

In line with the present study, Emandi et al. (2019) identified deletion on 3q29 chromosome band in preschool boy, first child of healthy non – consanguineous parents, showing particular NTD known as microcephaly. Besides, Emandi et al. (2019) pointed out that the deletion of 3q29 related with cleft lip and palate defect. On contrary to, Pollak et a. (2019) depicted that deletion 3q29 is spontaneous, hence it is not inherited. To this end, the neonatal NTDs identified in the present study, may not be correlated with 3q29 deletion in maternal chromosomes. However, the present finding is congruent with the current finding that deletion on 3q29 chromosomal band was detected in 2.17% of the study populations. It might not contribute to the development of NTDs (Pollak et a., 2019).

Micro deletion involving the gene-rich subtelomeric region of the long arm of chromosome located on 9q34.3 is a rare condition, even though the incidence of 9q34.3 deletion is not yet well established. The increasing number of patients reported with 9q34.3 is most likely related to the extensive clinical application of telomere FISH. Consequently, this micro deletion syndrome may be more common than previously assumed (Yatsenko et al., 2005; Cormier-Daire et al. 2003; Anderlid et al., 2002; Ayyash et al., 1997). A monogenic cause and autosomal recessive mode of inheritance have been considered to be probably associated with a normal karyotype (Yatsenko et al., 2005). Differently, a number of findings on patients with multiple CAs with chromosomal imbalances of 3p, 3q, 9p, or 11q are heterogeneous conditions with micro deletion (McGaughran et al., 2000). In the present investigation, the deletion of 9q34.3 involved specific gene EHMT1 using MLPA analysis. In contrary, Yatsenko et al. (2005) identified additional overlapping gene CACNA1B. According to Yatsenko et al.(2005) report, the deletion sizes varied from 700 kb to 2.3 Mb. The same authors also, reported that the 700 kb smallest region two overlaping genes, EHMT1 and CACNA1B, were potentially associated with the phenotypic features in 9q34.3 monosomy.

In this finding, deletion of 15q26.3 in the region of candidate gene TM2D3-3 was identified. Besides, the TM2D3 located in the deleted region of 15q26.3 of the chromosomal band synthesizes a protein that has a structural module linked to the transmembrane domain G protein coupled receptor superfamily and may have regulatory roles in cell death or proliferation signal cascades (Davidsson et al., 2008; Mahr et al., 2006). Thus, it might be related to the development of CAs if inherited to the developing fetuses. Congruent to our findings, several scientific reports showed terminal deletion of 15q26.3 (Biggio et al., 2004; Bhakta et al., 2005; Klaassens et al., 2005).

Five candidate genes causing the phenotype were within the deleted region, i.e. IGF1R, NR2F2, MEF2A, MCTP2, and CHD2 (Davidsson et al., 2008). However, a single candidate gene (TM2D3) is detected in our findings. The difference might be because of the approach and methods we used. In the present case, MLPA was used.

The present findings also point out that microdeletion occurred on the short arm of chromosome 15 located in the region of 15p11.2 within NDN1 candidate gene. Micro deletion of 15q11.2 may occur accidentally for the first time in an affected individual (*de*

novo mutation), or it may be inherited from a parent with the microdeletion. A blood test to look at the parents' chromosomes is needed to find out how the microdeletion occurred. A parent with the microdeletion has a 50% chance with each pregnancy to pass on the microdeletion (Cox and Butler, 2015). This report supports the current finding of microdeletion of 15p11.2 but with different candidate gene.

In this study, deletion of the short arm of chromosome 18 at specific chromosomal band 18p11.32 in 1 (2.17%) of study sampled populations was observed. Similarly, Cozaru et al. (2016) and Srebniak et al. (2011) reported the submicroscopic abnormality of chromosome 18p11.32 from phenotypically normal fetuses carrying a maternally-inherited interstitial deletion of 18p11.32. According to Srebniak et al. (2011) microdeletions were found with aneuploidy at 1,7 Mb deletion.

Chromosome 18p trisomy and partial 18p monosomy are referring to chromosomal abnormalities resulting from the deletion or duplication of a part of the short arm of chromosome (Srebniak et al., 2011; Turleau, 2008*).* It was indicated that partial 18p trisomy is rare (Misceo et al., 2009).

Reports indicated the de novo terminal 535 kb 18p11.32 deletions where the deletion include eight genes (USP14, THOC1, COLEC12, CETN1, CLUL1, C18orf56, TYM and ENOSF1) (Le Gall et al., 2017). In line with the report from the previous study, this finding indicated that the 18p11.32 deletions included THOC1 gene. The present study detected the deletions of 19q13.43 in 2 (4.35%) of the sampled population. The deletion of 19q13.43 includes the candidate CHP2A gene in sub telomere regions of the targated chromosome.

MLPA for Xq28 (SALSA P070-B2 probe mix) in the present study showed the deletions including VAMP7 gene. In contrary to the present findings, reports from other study showed de novo duplication of Xq28 which include the gene MECP2 and VAMP7 revealing those genes are susceptible to disorders (Antonio-Arce et al., 2016).

In the present finding, the sub telomers detection of targeted chromosome using P070 probe mix SALSA- MLPA showed duplications of 3p26.3, 6p25.3 on short arms of chromosomes 3 and 6 on specific genes of CHL1 and IRF4, respectively. Both duplications constitute 2.13 % the sample population.

In agreement with the present finding, recent studies reported that the duplication and deletion of 3p26.3 with specific gene CHL1 were identified among intellectually disabled individuals with epilepsy (Shoukier et al., 2013; Weehi et al., 2014; Li et al., 2016). The cell holding molecule L1-like (CHL1 or CALL) gene is located on chromosome 3p26.3 and is highly expressed in the central and peripheral nervous systems (Li et al., 2016). The protein encoded by CHL1 is a member of the L1 family of neural cell adhesion molecules, and plays a vital role in the development of the nervous system (Li et al., 2016).

In this study, duplications on the long arms of 5q35.3, 6q27, 11q25, 13q12.11, 15q26.3, and 22q13.33 were identified in specific genomic region of GNB2L1, TBP-2, IGSF9B-20, PSPC1, TM2D3-3, and UCKL1-7 respectively in a single individual 1(2.13%) of sample population. Duplications occur because of over expressions of normal protein related to the target gene (Antonio-Arce et al., 2016).

Strength of the study

The data collectors were trained health professionals, including gynecologists so that there were no case missings. The cases were evaluated from a large number of populations from which 35080 deliveries were attended within the period of two years of the study period. Case-control study design was used with a maximum case to control ratio of 1:4. Another strength of this study is that it uses both quantitative and experimental study design and involved secondary and primary data collection methods. MLPA analysis method was used for the genetic study which is a highly perceptive, forceful and high throughput method and can discriminate between deletion, duplication of genes and point mutations.

Limitations of the study

From the existing hospitals in the study regions, only six selected hospitals were used for the study. Delivers occurred at private hospitals were also not included. Secondly, the investigation was hospital-based study design and might have missed some cases of CAs for outside the study hospitals within the population. Thirdly, though structured data collection tools were used, some participants failed to give precise informations for some variables such as monthy income and educational level. A poor quality of data records in the study hospital, especially in retrospective, document review study was anothor drawback. As the study was hospital – based, the incidence rate may not represent the actual incidence of CAs in the total population.

Chapter Six

6. Conclusion and recommendation

6.1 Conclusion

The present study revealed that the prevalence of the CAs at birth was 5.5 cases per 1000 births. Among twenty-one CAs identified, NTDs were the most prevalent, while all the rest constitute only a quarter of the total CAs identified. The study also revealed that nearly equal proportions of CAs occurred among males and females. The majority of the mothers who gave birth to infants with CAs were younger than 35 years old. The most frequent CA was NTDs followed by musculoskeletal defects. Male newborns were relatively more affected than female newborns. More frequent newborns with CAs were prematurely delivered neonates. Most of the newborns with CAs were stillbirth. From the frequency distribution of associated factors, mothers who had neonates with CAs were exposed to passive smoking, pesticides, unidentified medicine during the first three months. Poor folic acid supplementation was more frequent among the neonates delivered with CAs.

Maternal socio-demographic and economic factors such as maternal educational level, average monthly income, maternal occupation, and paternal age showed no significant association with the occurrence CAs. Associated risk factors such as maternal passive smoking, exposure to pesticides, exposure to chemicals, use of unidentified medicine during the first trimester, use of surface water for drinking, and maternal birth history of CAs in the family had significant association for the occurrence of CAs. Maternal diabetes mellitus had a significant association with the risk of having neonates with CAs. The use of folic acid during the indexed pregnancy had a significant protective effect against the occurrence of CAs. There is a need to continuously provide health information for the community on how to prevent the occurrence of CAs and improve the quality of ANC as well as the folic acid supplementation mainly through food fortifications.

6.2 Recommendation

Based on the current findings, recommendations are forwarded to the concerned bodies.

To ministry of Health/regional health bureau/ policy makers:

- As the BDs are the leading causes of neonatal morbidity and mortality, prior planning, conniving and execution of anticipation and managing schemes are vital.
- Comprehensible BD/CA registry systems and policy need to be recognized at national, regional and district level.
- Develop strategies in documenting the need for the avoidance of CA.
- There is a need to continuously provide health information for the community on how to prevent the occurrence of CAs. To this effect, there is a need to advance the worth of ANC as well as the folic acid supplementation mainly through food fortifications.

To Health care providers:

- Special care for infants born with BDs need to be provide.
- Early identification of the defects and send them to appropriate specialties.
- Offer necessary condition for BDs genetic screening or genetic test and ascertain national and regional genetic counseling sectors.
- Education programs be supposed to counsel women to limit or avoid exposure to smoking and staying with smokers during early pregnancy.

To the community

Pregnant mothers need to enjoy ANC follow up during their pregnancies and need to use folic acid supplementation as per the health care providers' recommendation.

- Adequate education concerning the causes of BDs, possible risk factors and how to protect themselves during pregnancy are paramount inprotant.
- Women after the age of 35 need to be discouraged not to give birth so as to reduce the risk of chromosomal disorder.
- Parents of the children with BDs supposed to be guided to organizations that offer rehabilitation and psychosocial support.

To the researchers

- Further wider lare population-based study need to be conducted.
- Further study need to be conducted to investigate a wide range of associated factors for the occurance of CAs.
- As the presnt genetic study provides the start, further advanced genetics of birth defects study need to be conducted.

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8. Annexes

8.1 Annex I: Consent form and questionnaire (English, Amharic and Afan Oromo Version)

ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SHOOL OF MEDICINE

INFORMED CONSENT FORM

Investigations of Congenital Anomalies at Birth and Associated Risk Factors in South western Ethiopia

I, _____ (name), have read the information sheet above and clearly understood the purpose of the research. I have understood all that has been read and had my questions answered satisfactorily. I also understand that I can change my mind at any stage and it will not affect me in any way. I hereby need to assure with my signature below that I, without any coercion or forceful act by the research team, have decided to voluntarily participate in the study to contribute my part in the effort being made for the investigation of the congenital anomalies at birth and associated risk factors in southwestern Ethiopia.

Please *tick* I **agree to participate/ take part in this research**

□ **Yes**

□ **No**

Participant's signature:_____ Date:_____

Data collector's signature:_____Date:_____

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የጤና ሳይንስ ኮሌጅ

የስምምነት ፎርም

የአብሮ ወለድ ጉድለቶችና ተያያዥ የሆኑ አጋላጭ ምክንያቶች ጥናት በደቡብ ምዕራብ ኢትዮጵያ

እኔ _____ (ስም) ከላይ የተገለፀውን መረጃ አንብቤያለሁ፡፡ የምርምሩን አላማም በግልፅ ተረድቻለሁ፡፡ ያነበብኩትን በሙሉ የተረዳሁ ሲሆን የነበሩኝጥ ያቁዎችም በሚያረኩኝ መልኩ መልስ አግኝተዋል፡፡ እንዲሁም ምርምሩ እየተካሄደ ባለበት በማንኛውም ጊዜ ሃሳቤን መለወጥ የምችል መሆኑን እና ይህን ማድረጌም ምንም ጉዳት የማያስከትልብኝ መሆኑን ተረድቻለሁ፡፡ በጥናቱ ለመሳተፍም ከምርምር ቡድኑ ምንም አይነት ጫና ያልተደረገብኝ ሲሆን በደቡብም ዕራብ ኢትዮጵያ ለሚካሄደው አብሮወለድ ጉድለቶችን ና ተያያዥ የሆኑ አጋላጭ ምክንያቶችን ለማግኘት በሚደረገው ጥናት የራሴን ድርሻ ለማበርከት በፈቃደኝነት ውሳኔ አድርጌያለሁ፡፡

እባክዎ፡ ✓ ለመሳተፍ ይህንንም ልክት ያድርጉ

እስማማለሁ ☐

አልስማማም፡ ☐ እባክዎ የማይፈልጉ ከሆነ እዚህ ላይ ምልክት ያድርጉ

የተሳታፊው ፊርማ፡_____ ቀን፡_____

የመረጃሰብሳቢው ፊርማ፡_____ ቀን፡_____

UNIVERSIITII FINFINNEE

KOOLEJII SAAYINSII FAYYAA

WALIIGALTEE HIRMAATOTA WALIINII

Mata duree: **Dhibee da'imman waliin dhalatuu fi hiriiroo waantota dhibee kana fidan waliin qaban Kibba Iya Itoopiiyaatii**

Ani, _____ (maqaa), kaniin jedhamu odeefannoo armaan olitti ibsame dubbiseen/naa ibsamee hubadheera. Bu'aa qo'annichaa hubadheen jira. Kana malees gaaffiin gaaffadheef deebii ga'aa arga'een jira. Kanaafuu dirqii tokko malee qoranicha irratti hirmaachuuf walii galeen jira. Qo'annicha irratti hirmaachuukofis mallattooko armaan gadiin mirkaneessuun barbaada.

Hirmaachuukeef sanduqa armaan gadii keessatti mallattoo '√' barreessi.

Ni hirmaata: □ Eeyyee! □ Lakki

Malattoo Hirmaata: _____Guyyaa: _____

Maallatoo Daataa Funaana: _____ Guyyaa: _____

8.1Annex 2: Questionnaire

Addis Ababa University, College Health sciences, School of Medical, Department of Anatomy: Questionnaire format on accessing predisposing risk factors associated with birth defects in southwestern Ethiopia.

Instructions

Part A- Information about the neonate: Please fill the question by **marking 'x'** or by **writing the answers**

	Questions	**Response categories**	**Skip**
1	Date of data collection (DD/MM/YYYY) Guyyaa ragaan funaaname መረጃው የተሰበሰበበት ቀን	____/____/ ____	
2	Name of health institution Maqaa dhaabbata fayyaa የጤና ተቋሙ ስም	_____	
3	Card number Lakkofsa kaardii የካርድ ቁጥር	_____	
4	Date of birth (DD/MM/YYYY) Guyyaa daa'imni dhalate ህጻኑ የተወለደበት ቀን	____/____/ _____	
5	Sex of newborn Saala daa'ima dhalatee ልጁ የተወለደበት ቀን	|__| Male/ Dhiira / ወንድ |__|Female / Dhalaa/ ሰት	
6	Birth weight(Gram) Ulfatina (giraamaan) የልጁ ክብደት	|_____|	
7	Birth outcome	Alive birth / fayya dhalte/ በሕይወት የተወለደ	

	Bu'aa dhalate የወሊድ ሁኔታ	Still birth / du'aa dhalate/ ሙቶ የተወለደ	
8	Type birth Gosa bu'aa dhaloota የወልድ አይነት	____Single / qeenxee/ አንድ --------Twin / lakkuu/ መንታ	
9	Gestational age at birth [weeks] Umurii ulfi itti da'ame (torbaaniin) የእርግዝና ግዜ በሣምንት	|_____|	
10	Birth order of this infant Daa'imichi da'ima meeqafadha ስንተኛ ልጅ ነው	|_____|	
11	Onset of labor Akkata itti dhufiinsa ciniinsuu የአወላለድ ሁኔታ	|__| Spontaneous / tasa/በድንገት |__| Induced/kakkasuu/ማነቃቃት	
12	Mode of delivery Haala da,uumsaa የወላድ ሁኔታ	|__| Spontaneous Vertex/ |__| Caesarean Section	

Part B_ Mother Characteristics and related factors

	Questions	**Response categories**	Skip
1	Age of the mother Ummurii haadhaa የእናተ ዕድሜ	|_____|	
2	Maternal Education Sadarkaa barnoota haadhaa የትምህርት ደረጃ	|__| Illiterate / kan hin baranne/ ያልተማረ If literate, grade |_____|	
3	What is your (the mother's) occupation? Hojii haadha የእናተ የሥራ ሁኔታ	|_____|	

4	Residential area (Zone/district) Iddoo jireenyaa የመኖሪያ አካባቢ	|_____/_____|	
5	Religion Amantii □□□□□	|__| Muslim |__| Orthodox |__| Protestant Other specify	
6	Source of drinking water: Madda bishaan dhugaatii የመጠጥ ዉሀ ሁነታ	|__| Pipe water/ bishan bombaa/የቧንባ ውሃ |__| Underground water / lafa jala/የጉድጓድ ውሃ |__| Surface water/bishan lagaa/ የወንዝ ውሃ Others	
7	Parity		
8	Gravid		
9	History of abortions Seenaa ulfa ofirraa baasuu ከዚህ በፊት ውርጃ ያውቃሉ	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
10	History of still births Seenaa daa'ima du'e da'uu የሞተ ልጅ ተወልዷል	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
11	Pervious history of congenital abnormality Kanaan dura seenaa "congenital abnormality" አካል ጉደለት ከዚህ በፊት ተወልዷል	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
13	Is there a history of congenital malformation in your family? Seenaan 'congenital malformation' maatii keessaan keessa jiraa? በቤተሰብ ውሰጥ "congenital formation" ተፈጥሮ ያውቃል	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
14	Age of the father		

	Umurii abbaa የአባት እድሜ	|_____|	
15	Average monthly income of the family Giddu gala galii ji'aa maatii የቤተሰብ ወርሀዊ ገቢ	|_____|	

Part C_ The index pregnancy, ask the mother the following questions regarding the index pregnancy (last pregnancy)

	Questions	**Response categories**	**Skip**
1	Did you attend antenatal care during the index pregnancy? Yeroo ulfa kee isa asa ammaa hordoffii da'uumsa duraa gootee turtee? የወሊድ ክትትል አደረገው ያውቃሉ	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
2	If yes, the number visits Yoo eeyyee ta'e, yeroo meeqa? አዎ ከሆነ ስንት ጊዜ	|_____|	
3	Did you use folic acid during the index pregnancy? Yeroo ulfa kee isa ammaa foolik asiidii fayyadamtee? በእርግዝና ጊዜ ፎልክ አሲድ ተጠቅመው ያውቃሉ ?	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
4	Did you [the mother] take alcohol during pregnancy? Yeroo ulfa kee alkoolii fayyadamtee beektaa? በእርግዝና ጊዜ አልኮል ተጠቅመው ያውቃሉ	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
6	Did you smoke cigarette during pregnancy? Yeroo ulfaa tamboo xuuxxee beektaa? በእርግዝና ጊዜ ሲጋራ ታጫሻለሽ ?	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
7	Were you staying with the cigarette smoker in the same room during pregnancy, hence passively smoking? Yeroo ulfa kee namoota tamboo xuuxan waliin turtee? በእርግዝና ጊዜስ ሲጋራ ከሚያጨሱ ሰዎች ጋር ሁነሽ ታውካለሽ	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
8	Were you exposed to X-ray during pregnancy?	|__| Yes /eeyyee/አዎ	

	Yeroo ulfakee rajii kaatee beektaa? በእርግዝና ጊዜ X-rey ተነስተው ያውቃሉ	| __ | No / lakki/ አይደለም	
9	If yes, at what estimated gestation age? Yoo eeyyee ta'e, yeroo tilmaama ummurii ulfaa ammamitti? አዎ ከሆነ በስንተኛው እርግዝና ዕድሜዎ	| _____ |	
10	Is there history of exposure to Pesticides?	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
11	Do you have epilepsy? Dhukkubaa gagabdoo(lubaabee) qabdaa? የአዙሪት በሽታ አለቦት ?		
12	If yes, did you use anti epileptic drugs during pregnancy? Yoo eeyyee ta'e, yeroo ulfa kee qorichaa farra gagabdoo fayyadamtaa? አዎ ከሆነ በእርግዝና ጊዜዎ መዳኒቱን ወሰደው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
13	Are you diabetic? Dhukkuba sukaara qabdaa? የስኳር በሽታ አለቦት	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
14	If yes, do you use medication regularly Yoo eeyyee ta'e, qoricha isaa idileen fayyadamtaa? አዎ ከሆነ መዳሀኒቱ በትክክል ተጠቅመዎል	| __ | Yes /lakki/አዎ | __ | No / lakki/ አይደለም	
15	Did you have any infections during pregnancy? Yeroo ulfa kee dhukkubsattee beektaa? በእርግዝና ጊዜ በበሽታ ተይዘው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
16	When was the infection during pregnancy Yeroon ulfaa dhukkubsatte yoom ture? በእርግዝና ጊዜሽ አሞሽ ያውቃል	| _____ |	
17	What was it Yoo eyyee ta'e maal inni አዎ ከሆነ ምን ዓይነት በሽታ	| _____ |	

19	Did you use antibiotics? Qorichaa farra baakteeriyaa fayyadamtee? ፀረ-ባክተሪያ መዳሃኒት ውስደው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
20	Did you [the mother] take anti retrovirus drugs (ARVs) during pregnancy? Yeroo ulfaa qoricha farra HIV fudhatee beektaa? በእርግዝና ጊዜ ፀረ HIV መዳሀኒት ውስደው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
21	If yes in above, mention the type/types of anti retrovirus drugs taken Yoo eeyyee ta'e gosa isaa ibsi? አዎ ከሆነ ዓይነቱን ይንገሩን		
22	For how long did you take anti retrovirus drugs? Qorichaa farra HIV kana hammamiif fudhate? ፀረ HIV መዳሀኒት ለምን ያህል ጊዜ		
23	Do you have asthma? Dhukkuba asmii qabdaa አስም አለቦት	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
24	Did you take a drug during the first three months of the pregnancy? Yeroo ulfa kee ji'oota sadan duraaf qoricha fudhattee beektaa? በመጀመሪያ ሶስት ወራት መዳሀኒት ወስደው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
25	If **yes**, please specify the name of the drug(s) Yoo eeyyee ta'e, qorichicha nuuf ibsitaa? አዎ ከሆነ ምን ዓይነት መዳሀኒት ነው	| _____ |	
26	Did you take a drug between the 4th and 6th months the pregnancy? Ji'a ulfa kee 4ffaa fi 6ffaa gidduutti qoricha fudhatee beektaa? በአራተኛው እርግዝና ወራት ውስጥ መዳኒት ወስደው ያውቃሉ	| __ | Yes /eeyyee/አዎ | __ | No / lakki/ አይደለም	
27	If **yes**, please specify the name of the drug(s) Yoo eeyyee ta'e, qorrichichaa nu ibsi	| _____ |	

	አዎ ከሆነ የመዳኒቱን ዓይነት ገለፁልን		
28	Did you take a drug(s) during the last three months of the pregnancy? Ji'oota sadan dhuma yeroo ulfa keetti qoricha fudgattee beektaa? በመጨረሻው ሶስት የዕርግዝና ወራት ውስጥ መዳኒት ወስደው ያውቃሉ?	|__| **Yes** /eeyyee/አዎ |__| **No** / lakki/ አይደለም	
29	If **yes**, please specify drug(s) Yoo eeyyee ta'e, qorichicha nuu ibsi አዎ ከሆነ የመዳኒቱን ዓይነት ይንገሩን	|_____|	
30	Did you drink coffee during pregnancy Yeroo ulfaketti buna dhugaa turtee? ቡና በዕርግዝና ጊዜ ይጠጡ ነበረ	|__| Yes /eeyyee/አዎ |__| Nolakki/ አይደለም	
31	Did you chew khat during pregnancy? Yeroo ulfaketti caatii qaamaa turtee? ጫት በዕርግዝና ጊዜ ይቅሙ ነበረ	|__| Yes /eeyyee/አዎ |__| No / lakki/ አይደለም	
32	Do you have hypertension disorder? Dhibee dhiibaa dhiigaa qabdaa? የደም ግፊት በሽታ አለቦዎት	|__|Yes /eeyyee/አዎ |__|No/ lakki/ አይደለም	

8.3. Information about the presence and types of congenital anomalies

Congenital malformation		
Are there congenital anomalies	|__| Yes |__| No	
If yes, what types of birth defects or anomalies does the child have? Mark 'x'		
Central Nervous system 1. |__| Hydrocephalus 2. |__| Meningomyelocele 3. |__| Microcephaly 4. |__| Anencephaly 5. |__| Encenphocele 6. |__| Craniorachischisis 7. |__|Spina bifida 8. Others_____		
Gastrointestinal defects 1. |__|Trachea-esophageal fistula 2. |__|gastroschisis 3. |__|Diaphragmatic hernia 4. |__|Duodenal atresia 5. |__|Imperforate anus 6. |__|Ectopic anus 7. |__|Congenital inguinal hernia 8. |__|Umbilical hernia 9. |__|Epigastric hernia 10. Others_____		

Genitourinary defects

1. |__| Amibigious genitalia
2. |__| Urethral fistula
3. |__| Meatal stenosis
4. |__| Penile tourniquet
5. |__| Hypospadias
6. |__| Epispadias
7. Others_____

Musculoskeletal defects

9. |__| Club foot: bilateral _____ unilateral
10. |__| Ankyloglossia
11. |__| Palatine fistula
12. |__| Clefet lip only: median ____,bilateral_____, unilateral___
13. |__| Cleft palate only: median ____,bilateral_____, unilateral___
14. |__| Cleft lip with palate: median ____,bilateral_____, unilateral___
15. |__| Fistula in the neck
16. |__| Congenital torticolis
17. Others:_____

Data Collector

a. **Name:** _____
b. **Date:** _____

Signature: _____

9 Appendix

Apparatus and materials used in the laboratory protocol

- Heparin tubes
- Microcenterifuge tube
- Incubator at 56^0C
- DNeasy Mini spin column
- 2 ml collecting tube
- Centrifuge at ≥6000 x g (12000 rpm)
- Nanodrop spectrophotometry WPA,Cambridge, UK
- PCR machine
- Thermocycler
- Strips or plates of 0.2ml
- Agaros gel plate
- Capillary electrophoresis machine

I. Computer software used in the protocol

- Coffalyser software, version: v.140721.1958 for fragment and comparative analysis

II. Chemicals used in the protocol

a. Buffer used

- 200 µl buffer AL (Lying buffer)
- 500 µl buffer AW1 (wash buffer 1)
- 500 µl Buffer AW2 (Wash buffer 2)
- 200 µl Buffer AE (Elution buffer)
- TE Buffer (PH 7.5)

b. Enzymes used in the protocol

- proteinase K
- Ligase – 65 master mix
- Ligase buffer A
- Ligase buffer B
- Polymerase

c. Alcohol

- Ethanol alcohol (96- 100%)