CYTOGENETIC ANDMOLECULAR GENETIC STUDIES OF CHILDREN WITH DOWN SYNDROME AND ASSOCIATED RISK FACTORS IN KANO STATE, NIGERIA

BY

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DECLARATION

I Muhammad, Abdulhakeem Miko hereby declare that the work in this thesis titled**"Cytogeneticand Molecular genetic studies of children with down syndrome and associated risk factorsin Kano state, Nigeria**" was performed by me in the department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, ABU, Zaria, under the supervision of Dr. S. A. Musa, Dr. J.A Timbuak and Prof. (Mrs.) J.O. Anyiam.The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree at any institution.

_____ _____ _____

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CERTIFICATION

This thesis entitled "***Cytogenetic and Molecular genetic studies of children with down syndrome and Associated risk factorsin Kano state, Nigeria***" by Muhammad, Abdulhakeem Miko meet the regulation governing the award of Doctor of Philosophy (Ph.D.) degree in Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria, under the supervision of Dr. S. A. Musa, Dr. J.A. Timbuak, and Prof. (Mrs.) J.O. Anyiam. It is therefore approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to my daughterFareeha Miko and her mother RaliyaYahyaZimit

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDIXES

LIST OF ABBREVIATIONS

ALL- Acute Lymphoblastic Leukemia

AMKL- Acute Megakaryoblastic Leukemia

ATP- Adenosine Triphosphate

AVSD- Atrioventricular Septal Defects

ASD- Atrial Septal Defect

AUC- Area Under Curve

BP- Basepair

CHD- Congenital Heart Defect

CGS- Centre for Genetic Studies

CM1A- Charcot Marie Tooth Diseases Type 1A

DCR- Down Syndrome Critical Region

DNA- Deoxyribonucleic Acid

DS- Down Syndrome

DTU- Diarrhoel Training Unit

EPU- Emergency Paediatric Unit

EUROCAT- European Registration of Congenital Anomalies and Twins

FCC- Family Care Centre

FGN- Federal Government of Nigeria

GTG- Giemsa Banding

HNPP-Hereditary Neuropathy with Liability to Pressure Palsy

HBPH- Hasiya Bayero Pediatric Hospital

ID- Identification Number

IQ- Intelligent Quotient

ISCN- International System for Human Cytogenetic Nomenclature

MAKAUT- Maulana Abul Kalaam Azad University of Technology

MMSH- Murtala Muhammad Specialist Hospital

MI- Maternal MeiosisI

MtDNA- Mitochondrial Mutations

MTHFR- Methylenetetrahydrofolate Reductase

Nacl- Sodium Chloride

NDJ- Non- disjunction

NPC- National Population Commission

PHA- Phytohaematogglutnin

PCR- Polymerase Chain Reaction

ROC- Receiver Operating Curve

SCC- Sister Chromatid Cohesion

SCU- Sickle Cell Unit

SMS- Smith- Magenis Syndrome

STR- Short Tandem Repeats

USA- United State of America

ABSTRACT

Down syndrome (DS) is the most common type of chromosomal trisomy found in the newborn. It is associated with hypotonia, delayed development, mental retardation and characteristic facial features.This study of cytogenetic analysis was conducted toconfirm the clinical diagnosis of Down syndrome (DS)and to evaluate the risk factors associated with trisomy 21 in a group of subjects in Kano State of Nigeria.The study alsodemonstrate sensitivity of DNA diagnosis of Down syndrome using polymerase chain reaction (PCR) and short tandem repeat (STR) markers, and to determine the origin of the non-disjoined chromosome 21. The study further determined the relationship between the socio-demographic data with the presence of CHD in DS subjects. DS patients (=35) were recruited for the study, but 16 DS subjects comprising of 9 males and 7 females with sex ratio was 1.3:1 were randomly selected for karyotyping analysis. GTG-band was done according to the standard protocols.Molecular analysis was carried out by PCR based method, using polymorphic microsatellite markers D21S11 situated on the long arm of the chromosome 21 at 21q21. The amplified products were subjected to 8% polyacrylamide gel electrophoresis and alleles were scored by staining with ethidium bromide. Pearson's Chi square test, independent sample, and receiver operating characteristic curve,box and whisker plots were used to analyze the data. Probability - value ($p< 0.05$) was set as level of significance used to analyze the data. The results show that among the 16 cases with DS selected for karyotyping, all of them (100%) had free trisomy 21 with no translocation and mosaic DS.Trisomy 21 was detected by the presence of three distinct alleles and transmission of alleles from in all DS families. The STR marker D21S11 was able to detect 100% cases of trisomy 21 and also emphasize the fact that trisomy 21 was due to meiotic errors in maternal chromosomes.Age diagnosis was 60% in DS children < 1 year, 32% in DS between 1-2 years and 8% in DS > 2 years. Birth order was an important risk factor associated with trisomy 21, 21% of affected

children were of last or second last birth order.Of the 35 DS subjects, the prominent craniofacial features noted were upward slanting fissures(17.5 %), facial profile (17.5%), low set ears (17.5 %), simian crease (17.5%), flat occiput in 35 of DS (17.5%), macroglossia (19%) in 33 and short neck (33%), microcephaly in 34 (17%of) DS subjects, congenital heart disease was diagnosed in 8 cases (4%).Hypothyroidism in 2 subjects (1%), pes planus was seen in 1 DS subjects (0.5%). A total of 35 cases, 29 (14.5%) had documented hypotonia. The prevalence of CHD in 35 DS children reviewed,20% had an associated CHD while 80% had no CHD. The most common isolated cardiac lesion was ventricular septal defect (VSD), found in 57% patients, and followed by atrioventricular septal defect (AVSD) in 43%. The mean maternal age at birth of the affected children was 35.66 ± 8.593 years. It was significantly higher than mothers of non-trisomic children (31.28 ± 5.96 years; p = 0.0002). There was a significant difference in the maternal and paternal ages of patient with DS and non trisomic subjects. Children with free trisomy of chromosome 21 are more frequently born to mothers older than 35 years of age.In both maternal and paternal mean ages of DS patients, the mean was higher compared to that of control. Maternal age was a significant (AUC= 0.67, p=0.025) risk factor of giving birth to children with DS.A cut-off value of 42 years and above indicates the risk of giving birth to child with DS (Sensitivity = 0.31, Specificity= 1.00).However, the paternal age had no effect (AUC= 0.62, p=0.132) as a risk factor of giving birth to DS subject, but 47 years of age was found to have the best sensitivity and specificity in discriminating paternal age of DS birth (Sensitivity = 0.51, Specificity= 0.84).Among the eight DS with CHD, it was found that 3% of male DS cases had CHD, whereas only 5% of female cases had CHD.Confirmation of clinical diagnosis by the identification of specific types of chromosomal abnormalities in DS children is very important as it will be used to create awareness about the recurrence risk of having children trisomy 21.The

PCR-based DNA diagnostic method using STR was found to be sensitive, reproducible, and efficient, not only for diagnosis of trisomy 21, but also for tracing allelic transmission from parents to the offspring. This method can also be employed in the diagnosis of trisomy 13 and 18.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Down syndrome (DS) is one of the most common chromosomal causes of intellectual disability, found in 1 in every 691 (Kucik *et al*., 2013; CDC, 2014) or 733 babies (Zingman, 2013). The genetic cause of the disease, clinically described in 1866 by John Langdon Down, was discovered in 1959 by Jerome Lejeune (Zingman, 2013). It is caused by a triplicate state (trisomy) of all or a critical portion of chromosome 21.

Down syndrome subjects have multiple morphological and functional alterations of their body structures, from cellular organelles to multiorgan systems, with varying degrees of gravity (Roizen and Patterson, 2003; Zingman, 2013). Due to the various congenital anomalies and to some higher incidence of postnatal diseases, persons with Down syndrome had mortality 5–11 times higher than the general population (Zhu *et al*., 2013). Surgery (especially correction of congenital heart defects) and medical management of Down syndrome subjects had modified their life expectancy, and more recent birth cohorts have lower mortality rates than older ones (Rankin *et al*., 2012). In the USA, Kucik *et al* (2013) reported survival probability rates of 98% at 1 month of age, 93% at 1 year, 91% at 5 years, 88.9% at 15 years, 88% at 20 years and 87.5% at 25 years of age (Kucik *et al*., 2013; Zingman, 2013). In Italy, approximately 10,500 subjects with Down syndrome are aged 0-14 years, 32,000 are between 15 and 44 years and 5,000 are older than 44 years; most Italian persons with Down syndrome are expected to live until 45-46 years, with a 13% survival rate between 45 and 65 years of age (Arosio *et al*., 2004).

Down syndrome can be found in babies from ethnic groups all around the world; a recent report found that mortality risk in subjects with Down syndrome is partially influenced by ethnicity, with lower survival rates of non-Hispanic black children compared with non- Hispanic white children living in the same area (Kucik *et al*., 2013). Some kind of heterogeneity in the expressions of Down syndrome alterations in the various ethnic groups may contribute to explain this finding. Indeed, facial characteristics in Down syndrome subjects seem to be nearly the same all over the world, but detailed quantitative analyses of the ethnic groups are only partial, with data available only for the United States (Farkas *et al*., 2001a, b, 2002a, b), Spain (Quintanilla *et al*., 2002), Croatia (Bagic and Verzak, 2003), Italy (Ferrario *etal*., 2004; Sforza *et al*., 2004, 2012a, b), Southern India (Asha *et al*., 2011) and Northern Sudan (Sforza *et al*., 2011a, b, 2012b).

Human Chromosome 21 was first mapped in May 2000 (Hattori, 2000). In general, this leads to an over expression of the genes (Rong *et al*., 2005). Understanding the genes involved may help to target medical treatment to individuals with Down syndrome. It is estimated that chromosome 21 contains 200 to 250 genes. Research has identified a region of the chromosome that contains the main genes responsible for the pathogenesis ofDown syndrome, located proximal to 21q22.3. The search for major genes involved in Down syndrome characteristics is normally in the region 21q21–21q22.3 (Rahmani *et al*., 2005).

Basic cytogenetically screening ("karyotyping") is accepted as standard diagnostic procedure for DS detection, as prenatal and postnatal diagnostics. Karyotyping is based on analysis of numerical and structural changes of all observed chromosomes. However, this method could not

be used for detection of microdeletion and microduplication. In addition, karyotyping it time consuming method which requires significant time period for sampling, preparation and analysis of metaphase chromosomes. Therefore, last few years, with more or less success, various molecular-genetic analysis was introduced as alternative method. Detailed examination and extensive application of the microsatellite regions, especially short repetitive DNA sequences (Short Tandem Repeat - STR) promote this marker as possible solution as fast, accurate, cheap and simple genetic tool that could be used in DS detection (Crkvenak-Gornik *et al.*, 2004). Short tandem repeats (STRs) are easily typed, ubiquitous and polymorphic loci with high mutation rates. The tendency of the mass application of STR markers has clearly defined them as the molecular polymorphisms which are widely used in population, forensic and medical genetics. STRs are short sequences of DNA, normally of length 2-5 base pairs that are repeated numerous times at the particular locus. Number of repetitions varies from person to person. The real value of the application of these markers lies in the simplicity and rapidity of the process and the possibility of simultaneously testing of a large number of STR markers in the so-called multiplex STR systems, enabling an extremely high degree of individualization in identifying biological evidence. Also, these sequences, in addition to its wide application in forensic DNA analysis, have become very attractive as a subject of genetic research from a medical point of view, because it could be performed on the buccal swab sample and it could be associated with certain genetic disorders (Primorac *et al*., 2014).

With the recombinant DNA technology, a new set of tools became available to the study of origin and mechanisms of chromosomal abnormalities using DNA polymorphism analysis. In the beginning this kind of analysis used chromosome 21-specific DNA probes to detect restriction fragment length polymorphisms (Davies *et al*., 1984). The development of the polymerase chain

reaction (PCR) amplification technique (Saiki *et al*., 1988) enabled the identification of novel and highly informative classes of DNA polymorphisms in the human genome, the so-called microsatellites or simple sequence repeat (SSR) polymorphisms (Weber and May *et al*., 1989; Litt and Luty, 1989; Economou *et al*., 1990). Especially the multi-allelic and easily typified microsatellites have contributed to mapping of the human genome (NIH/CEPH, 1992; Weisenbach *et al*., 1992) and to non-disjunction studies (Petersen*et al*., 1991).

1.2 Statement of the Research Problem

There is paucity of data on cytogenetic studies on Nigerians withDS. This is due to near or completes absence of and cytogenetic profilesamong patient with DS patients in our health facilities where prompt and accurate diagnosis could be made. Thus, management of cases of DS is based largely on clinical manifestation. Therefore, the present study is based oninvestigating the cytogenetic profiling of cases of Down syndrome.

Karyotyping is based on analysis of numerical and structural changes of all observed chromosomes. However, this method could not be used for detection of microdeletion and microduplication. The prenatal and postnatal diagnosis of trisomy 21 by simple PCR based method using STR (short tandem repeats) amplification for detection of trisomy 21 of Nigerians with DS has not being effectivein our clinical settings.

Basic karyotyping is not molecular and, as such the method could not be used for the determination of parental origin of extra chromosome 21 in the diagnosis of children with DS.

The Multiple Marker Screening Test (MMST) of maternal serum has been used worldwide in the past decades to screen for pregnancies complicated by fetal DS. In cases of high risk based on MMST screening or advanced maternal age, genetic amniocentesis is recommended in order to perform fetal karyotyping. About 60 to 70% of fetal DS cases can be identified by MMST, with a 5% false positive rate. Many polymorphic DNA markers containing short tandem repeats (STR) located on chromosome 21 have been discovered (Mc Innis *et al*., 1993). These polymorphic DNA markers have been applied in genomic studies such as constructing genetic linkage maps of chromosome 21 (Warren *et al.*, 1989; Petersen *et al*., 1991). It was proposed that STR polymorphism could be applied to detect aneuploidies.

The interval between genetic amniocentesis and the completion of karyotyping is a period of anxiety for the family (Pertl *et al*., 1999). Therefore, scientists continue to seek nascent marker(s) or protocols in order to detect this aneuploidy more rapidly.

The most intensively studied etiological factor for the occurrence of trisomy 21 is advanced maternal age while other risk factors are less well established. About 25% of pregnancies carrying DSfoetuses survive to birth, and post birth data indicate greatly improved life expectancy in about 80% of them. So, there is a huge challenge for providing health care for subjects with DS.

1.3 Justification of the Study

Findings fromkaryotyping or chromosomal analysis may provideinformation necessary for the assessment oftrisomy 21 status. This may also provide the bases for hypotheses that may explain the ethnic-related differences in the mortality risk of DS subjects.

Confirmation of clinical disease by the identification of specific types of chromosomal abnormalities in DS children will be used to create awareness about the recurrence risk of trisomy 21. This study will throw light on the various risk factors like maternal age, paternal age and other genetic compositions etc. which are likely to influence the expression of congenital heart defects (CHD) in DS.

Findings from STR marker/s will help in detection of trisomy 21 by the presence of three alleles on the third copy of chromosome, thus confirming the cytogenetic analysis and will demonstrate the highly polymorphic microsatellite markers for the accurate determination of the origin of chromosome 21 in DS subjects.

From literature, this is the first report of associated risk factors, karyotype patternand PCR-based detection method using STR marker of children with Down syndrome in Kano State, Nigeria.

1.4 Aim and Objectives of the Study

1.4.1 Aim of the study

The aim of the study is evaluate cytogenetic profile and investigate sensitivity of DNA diagnosis of DS using simple PCR and STR markers for detection of trisomy 21 and also determine the

relationship between the socio-demographic data with the presence of CHD inchildren with Down syndrome in Kano metropolis.

1.4.2 Objectives of the study

The objectives of this study are to:

i. confirm the clinical diagnosis of DS attending clinic at Paediatric OutpatientsDepartment of Murtala Muhammad Specialist Hospital and Hasiya Bayero Paediatric Hospitalin Kano using cytogenetic analysis.
ii. describe chromosomalkaryotypes of DS patients attending clinic in the same hospitals.
iii. demonstrate sensitivity of DNA diagnosis of DS using DNA and STR markers for detection of trisomy 21.
iv. describe the socio-demographic data of children with DS and control
v. describe the clinical features of children with DS and control.
vi. determine the prevalence and pattern of congenital heart disease seen in DS subjects.
vii. Determine the mean maternal and paternal ages of DS mothers and control in Kano State.
viii. Determine the association between parental age and CHD in children with DSbased on sex and type and its correlation with clinical features in Kano State.
ix. Describe the frequency of free trisomy 21 type in DS children and its association with maternal and paternal age in Kano State. 3

1.5 Research Hypotheses

1. There is correlationship between maternal age and having a child with DS

2. There is correlationship between congenital heart defect and having a child with Down syndrome.

3. There is associationship between clinical features and clinical diagnosis of DS subjects

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Down's Syndrome: Historical Aspects

In 1866, the British physician John Langdon Down (1828-1896) published an article which described children with a common phenotype and with intellectual disability (Birch, 1973; Ward,1999). He accurately described the features of Down syndrome including hypotonia, mental retardation and facial features, and classical pattern of palmar creases of hands. He referred the name of Down syndrome people to "mongoloids" because of their upward slanting eyes which gave the impression of Mongolian people.

In 1961, the WHO informally recommended not to use the term Mongolism and to name it DS because some biomedical researchers were calling to stop the term "mongolism" and to describe people with DS as trisomy 21 anomaly (Howard-Jones, 1979). Already in 1932, Waardenburg hypothesized that non-disjunction which leads to trisomy or monosomy might be the cause of DS (Allen, 1974). Then, in 1959, the French geneticist Jerome Lejeune showed that DS is caused by a trisomy of chromosome 21 and his finding wassubsequently confirmed by a publication from Jacobs and her group (Jacobs and Baikie, 1959).

DS was first described in medical literature by John Langdon Down in 1866. During this era individuals with cognitive impairment (i.e. mental retardation) were often referred to as "idiots" and "imbeciles" and rarely differentiated into subcategories based upon differential diagnoses.

Using a hierarchical racial classification system that was popular during his age, John Langdon Down noted the resemblance of facial features among individuals with DS and individuals of Mongolian descent (Down, 1866; Volpe, 1986). Down also noted the characteristic facial appearance and shared phenotypic features of unrelated individuals with DS by arguing that when individuals with this condition are placed side by side, they appear to be siblings (Down, 1866). Based upon these observations Down concluded that individuals with DS differed from other types of individuals with cognitive impairment and labeled these individuals as "Mongolian idiots" or "mongoloids" (Down, 1866). Although other authors (e.g. Esquirol, 1838; Seguin, 1846) may have described individuals with DS before Down's publication in 1866, Down is credited with being the first person to group together individuals with DS based upon their phenotypic similarities to define a subcategory of individuals with cognitive impairment (Stratford, 1989; Stratford, 1996;Pueschel, 2000; Kava *et al*., 2004; Megarbane *et al*., 2009). While speculation and conjecture often attributed the cause of DS to alcoholism, syphilis, tuberculosis, occupational exposures, and even regression to a primitive human state (Pueschel, 2000), the true cause of DS – trisomy 21 - would not be proven until 1959 when LeJeune discovered that DS individuals have an extra copy of chromosome 21 (LeJeune and Turpin, 1959). However, after a thorough investigation of DS Benda (1941) dismissed altogether the idea of a racial mutation or atavistic regression to a previous human state as being the cause of DS (Benda, 1941). The hierarchical racial ladder of Down's era viewed the races of mankind as being fixed and definite, with Caucasians being superior to all other races and Mongolians being at or towards the bottom of the ladder (Volpe, 1986). Although Down's "Mongolian idiot" and "mongoloid" labels would be viewed as racist today, the use of these terms was a consequence of the prevailing ideas of racial hierarchies from his era (Volpe, 1986). By combining this

interpretative framework with his phenotypic observations of individuals with DS, down made an argument for the "unity of the human species" (Down, 1866). Down reasoned that if a disease can break down supposedly "fixed" racial barriers by producing a Mongolian-like child from non-Mongolian parents, then the racial categories of mankind are likely not fixed at all and quite variable (Down, 1866). This was an unpopular opinion at the time of Down's publication. Interestingly, If Down had not favored this hierarchical racial classification system for understanding differences between individuals with cognitive impairment, it is likely that it would have taken longer for medical scientists to classify DS as different from other forms of cognitive impairment. Today we know that DS is found in all ethnic backgrounds and socioeconomic statuses across the world at a frequency of about 1:700 (Evers-Kiebooms *et al*., 1985; Christianson *et al*., 1995;Christianson 1997; Fernandes *et al*., 2001; Kuppermann *et al*., 2006; CDCP, 2006; Azman *et al.*, 2007). On average more than 700 DS children are born each day worldwide and more than 255,000 individuals with DS are born each year. A marked maternal age effect has been noted (Shuttleworth, 1909; von Hofe, 1922; Penrose, 1951; Carter and Mac-Carthy, 1951;Boue *et al*., 1975; Hook, 1989; Freeman *et al.,* 2007). Risk of having a child with DS in women aged 35-39 years is 6.5 times higher than that of 20-24-year old, and that risk increases by 20.5-fold in women 40-44 years of age (Kava *et al*., 2004). However, most children with DS (63%) are born to younger mothers because younger mothers tend to produce more children than older mothers (Kava *et al*., 2004). A male to female bias of 1.15:1 has been noted for DS (Lyle *et al*., 1972; Byard, 2007).

Individuals with DS typically have intelligence quotients (IQs) in the range of 20-85 (Lyle *et al*., 1972; Byard, 2007). In the 1960's only 4% of individuals with DS learned to read and only 2% learned to write (Lyle *et al*., 1972), but these percentages are higher today. Due to improved

healthcare, the life expectancy for individuals with DS has consistently risen from 9 years in 1900, to 16-30 years in the 1960's, and to more than 50 years today (Collman and Stoller, 1963; Hall 1965; Roizen and Patterson, 2003; Megarbane *et al*., 2009), which has caused an increase in prevalence (Einfeld and Brown, 2010). However, the average lifespan of 9 years in 1900 may have been skewed due to a cultural tendency to institutionalize cognitively impaired individuals during this time, combined with the poor living conditions and developmental outcomes associated with many of these institutions (Kugel and Reque, 1961; Stimson *et al*., 1968; Kirman, 1976). It is possible that in some cultures individuals with DS who did not have severe health problems may have enjoyed a higher average lifespan (Stratford, 1982).

2.2 Types of Chromosomal Aberrations

Cytogenetically Down syndrome is divided into three types: free trisomy 21, translocation and mosaic trisomy 21. (Giraud and Mattei, 1975).

2.2.1 Regular or free trisomy 21

In this type of aberration, the carrier has 47 chromosomes, including three chromosomes 21. It accounts for nearly 90% of DS cases. Standard trisomy 21 typically occurs sporadically; therefore, the recurrence risk is low.

Most of free trisomy 21 cases (85-90%) originate from errors in maternal meiosis. Maternal meiosis I is the most frequently affected stage of nondisjunction (>75%),whereas maternal meiosis II errors account for >20%. In 5% of free trisomy 21, paternalmeiotic errors can be observed, here meiosis II nondisjunction is more frequent than meiosisI errors. In addition,

postzygotic mitotic errors have also been reported (5%). Thepredominant influence of disturbed maternal meiosis is reflected by the decreased number of chiasmata in meiosis I increasing with maternal age. Indeed, the reason for thisassociation is unknown, however numerous hypotheses have been proposed (Hulten et *al*., 2010).

All cells have an extra chromosome 21. Approximately 90-95% of individuals with Down syndrome have a free trisomy for chromosome 21 (Pangalos *et al*., 1994; Mutton *et al*., 1996; Savage *et al.,* 1998).

DS can result from distinct genetic anomalies: nondisjunction, translocation, and mosaicism. Nondisjunction is common in humans and causes aneuploidy in an estimated 10-35% of conception, many of which end in spontaneous abortion or produce children with cognitive impairment and/or birth defects (Freeman *et al*., 2007). Nondisjunction is the cause of trisomy 21 in approximately 95% of living individuals with DS across the world (Kava *et al*., 2004; Freeman *et al*., 2007; Azman *et al.,* 2007). Approximately 75% of first trimester and 50% of second trimester trisomy 21 conceptions are lost before coming to term (Roper and Reeves, 2006; Freeman *et al*., 2007).

Nondisjunction of chromosome 21 occurs when two chromosomes 21 cells fail to segregate by sticking to each other during anaphase - the period of the cell cycle when spindle fibers pull chromatids to opposite poles to ensure that each daughter cell gets an identical set of chromosomes (Petersen and Mikkelsen, 2000). This process of faulty chromosomal separation is called "nondisjunction" because the two chromosomes fail to "disjoin" like they are supposed to

during normal cell division. This faulty division produces two abnormal cells: one with three copies of chromosome 21 and one with a single copy of chromosome 21. The cell with a single copy of chromosome 21 is not viable; however, the cell with three copies of chromosome 21 is viable and is used as a template to create future cells, ultimately resulting in DS. Advanced maternal age is the only well-documented risk factor for increasing the likelihood of nondisjunction occurring (Penrose, 1951). Nondisjunction can occur during mitosis or meiosis. However, the likelihood of Nondisjunction of chromosome 21 occurring during the very first mitotic cell division after fertilization is very low and is reported to only occur in about 2.7-3.8% of all DS cases (Petersen and Mikkelsen, 2000; Freeman *et al*., 2007).

While nondisjunction can occur during the very first cell division after fertilization, it is more likely that nondisjunction of chromosome 21 occurs during either the first or second meiotic cell division of either the ovum (oocyte) in the mother or the sperm in the father. Current karyotype evidence from living individuals with DS indicate that the extra copy of chromosome 21 results from a maternal error in ova division 92.8-93.2% of the time, and from a paternal error in sperm division 4.1-5.5% of the time (Peterson and Mikkelsen, 2000; Freeman *et al*., 2007). Meiotic nondisjunction errors are more common in older parents and are usually maternal in origin (Mikkelsen *et al*., 1980; Antonarakis, 1991; Petersen *et al*., 1993; Peterson and Mikkelsen, 2000).

The trisomy 21 sex ratio (males/females) is 1.16 in fetuses and 1.15 in live births, which supports the hypothesis that sex ratios are either skewed at conception or become skewed during embryonic development through differential intrauterine selection (Huether *et al*., 1996). An

excess of males has been noted for cases of meiotic nondisjunction that are paternal in origin (Hassold *et al.,* 1984; Petersen *et al*., 1993). It has been hypothesized that mechanisms of paternal nondisjunction may cause the extra chromosome 21 to preferentially segregate with the Y chromosome, which would help account for the sex ratio bias towards males (Petersen *et al.* 1993; Huether *et al.* 1996).

It is easy to misconstrue the evidence above to indicate that meiotic errors of the ova are more common in females than males; however, it is more likely that sperm cells which are impaired by a chromosomal anomaly are just less likely to fertilize an egg before the millions of other karyotypically normal sperm cells that they are competing with. Thus, most of pregnancies resulting in trisomy 21 occur from an egg with nondisjunction of chromosome 21 and normal sperm because abnormal sperm cells are less able to compete with their normal counterparts when searching for an oocyte to fertilize. At approximately 95%, nondisjunction is by far the most frequent cause of trisomy 21 (; Hassold *et al*., 1993; Fisher*et al*., 1995).

2.2.2 Translocation trisomy

Translocations involve a transfer of genetic material from one chromosome to another and consequently join two otherwise separated genes. The frequency of various chromosomal rearrangements in the general population varies from 1/625 to 1/5,000 (Bandyopadhyay *et al*., 2002). Translocations can be classified into two different types: reciprocal and Robertsonian. Reciprocal translocations occur more frequently than Robertsonian (Bandyopadhyay *et al*., 2002). The major impact of translocations is that they can generate significant chromosome

imbalance during segregation at meiosis, predisposing the carrier to the birth of abnormal child, stillbirth, miscarriage or infertility.

The extra chromosome 21 is attached to another chromosome. Translocation trisomies account for 2-4 % of the DS cases. In almost all cases of translocation trisomy, one of the parents is carrier of a balanced Robertsonian translocation of the long arm of Chromosome 21 to the short arm of a D- or G-group chromosome (Pangalos *et al*., 1994; Mutton *et al*., 1996; Savage and Peterson, 1998). *De novo* Robertsonian translocation are rare, one between chromosome 14 and 21 t (14; 21) has been described originating from maternal germ cells (Petersen *et al*., 1991). In contrast, most translocations between the long arms of two chromosomes 21t (21; 21) are isochromosomes due to a duplication of (21q) rather than a result of a Robertsonian translocation (Antonarakis *et al*., 1990).

Many published translocation breakpoints have shown that direct disruption of a gene can lead to an associated phenotype (Bhalla *et al*., 2004; Bocciardi *et al*., 2005; Klar *et al*., 2005) but studies also showed that translocation breakpoints can cause an effect on genes several kilobases away. Examples include a t(2;8) (q31;p21) which affects the *HOXD* gene 60 Kb away from the chromosome 2 breakpoint in a patient with mesomelic dysplasia and vertebral defects (Spitz *et al.*, 2002) and a t(6;11)(q14.2;q25) translocation affecting the*B3GAT1* gene which lies 299 Kb centromeric to the chromosome 11 breakpoint in a patient with psychosis (Jeffries *et al*., 2003). The position effect phenomenon arises from the disruption of *cis*-acting regulatory elements such as promoters, enhancers and silencers which can be directly altered, distanced from the gene they influence or brought intoproximity of a gene not normally under their control when a

chromosome undergoes a rearrangement. These elements have been observed as far away as 1.1 Mb from the gene they regulate as in the case of the SOX9cre1 element which was identified upstream of the *SOX9* gene (Bien-Willner *et al*., 2007).

2.2.2.1 Reciprocal translocations

These involve breakage of at least two non-homologous chromosomes and exchange of the fragments. The incidence of reciprocal translocations is ~1/625 in the general population (Van Dyke *et al*., 1983). The first reciprocal translocation to be associated with human cancer is t (9; 22) which formed a shortened derivative chromosome 22, also known as Philadelphia chromosomes (Ph) (Nowell and Hungerford,1960; Rowley,1973).

Most reciprocal translocations are nonrecurring rearrangements except for the t (11;22) reported in more than 160 unrelated families (Fraccaro *et al*., 1980; Zackai and Emmanuel,1980; Iselius *et al*.,1983). Breakpoint studies on both 11q and 22q have demonstrated a common site for rearrangement (Edelmann *et al*., 1999b; Shaikh *et al*., 1999). It was later suggested that a highly specific Alu-mediated recombination in the breakpoints could be the cause in the translocation (Hill *et al*., 2000).

The breakpoint on chromosome 22 maps within a low-copy region-specific repeat(LCR22) that is also associated with the breakpoints seen in del (22) (q11.2) inDiGeorge/VCF syndrome (Edelmann *et al*., 1999a). The breakpoint on the long arm of chromosome 11 is consistently found between two genetic markers, in a genomic region of about 185–190 kb (Edelmann *et al*., 1999b; Shaikh *et al*., 1999), and then narrowed to a 190-bp region harboring an AT-rich repeat

(Edelmann *et al*., 1999b; Kurahashi *et al*., 2000). The repetitive sequence found in the low-copy repeats on 22q also has multiple copies of an AT-rich sequence (Edelmann *et al*., 1999b). These findings suggest that AT- rich regions may be prone to recombination events that lead to rearrangements.

2.2.2.2 Robertsonian translocations

Robertsonian translocation (abbreviation rob) is named after the American insect geneticist, W.R.B. Robertson, who first described the translocation in grasshoppers in 1916. They are found in ~1 in 1,000 individuals (Hamerton *et al*., 1975; Blouin *et al*., 1994) making it the most common, recurrent structural rearrangements in humans. These translocations involve exchanges of the whole arm of acrocentric chromosomes (13-15, 21 and 22). The result is one long chromosome with a single centromere and the total number of chromosomes is reduced to 45. In the short arm, the p11 includes satellite DNAs I, II, III, IV and β; the p12 region contains multiple copies of the genes coding for the 18S and 28S ribosomal RNA (nucleolar organizer region); and the p13 region terminates with β- satellite DNA and telomeric sequences (Page *et al*., 1996; Bandyopadhyay *et al*., 2002).

Thus, the short-arm regions of the five pairs of human acrocentric chromosomes have extensive sequence homology although some sequences are not common to all acrocentric chromosomes (Bandyopadhyay *et al*., 2002).

Although all acrocentric chromosomes can participate in the translocation, the distribution of chromosomes was shown to be non-random (Therman *et al*., 1989). Rob (13q;14q) and

rob(14q21q) are the most common, constituting ~85% of all Robertsonian translocations (Therman *et al*., 1989). In ~50% of Robertsonian translocations, the rearrangements occur *de novo* (Shaffer *et al*., 1992) and in ~95% of the *de novo* cases, rob(13q14q) and rob(14q21q) originate during maternal meiosis (Page and Shaffer, 1997).

2.2.2.3 Deletions

Deletion involves loss of part of a chromosome either terminal or interstitial, resulting in monosomy in that region of the chromosome. Deletion therefore is an unbalanced rearrangement. It can involve deletion of a single base to an entire piece of chromosome (Lewis, 2005). In most cases, large cytogenetically visible deletions cause embryopathy which presents after birth as mental retardation, growth failure and multiple malformations.

In humans, cytogenetically visible autosomal deletions have a live birth incidence of about1 in 7,000 (Jacobs *et al*., 1992). The first chromosomal deletions identified in humans were the deletion of distal 5p associated with the cri-du-chat syndrome (Lejeune e*t al*., 1963; Lejeune *et al.,* 1964) and the distal deletion of 4p, subsequently named the Wolf- Hirschhorn syndrome (Wolf *et al*., 1965).

A principal method of producing deletions is by unequal crossing-over between region specific low copy-number repeat sequences that flank the deleted regions (Lupski *et al*., 1996; Chen *et al*., 1997; Lupski, 1998; Shaikh *et al*., 2000).

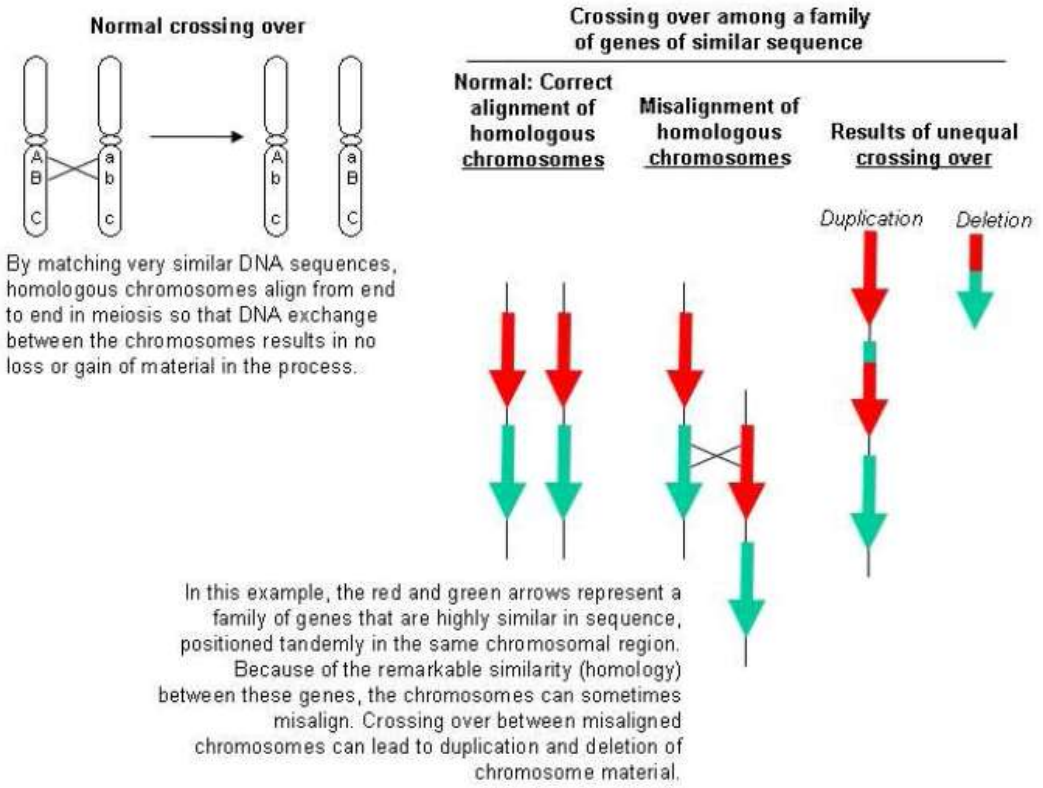


Figure 2.1: Unequal crossing over results in deletion and duplication rearrangements (Adapted from Nussbaum, McInnes and Willard, 2001).

2.2.2.3.1 Terminal deletions

Even though terminal deletions have been identified for all human chromosomes, only a few have a significant incidence. Common terminal deletions include 1p-, 4p-, 5p-, 9p-,11q-, 17p-, 18q-, and 22q- (Shaffer and Lupski, 2000). Terminal deletions usually do not occur at a single site but involve breakpoints at various regions with variable sizes (Shaffer and Lupski, 2000). However, some cases of terminal deletion demonstrated clustering at many locations (Christ *et al*., 1999; Wu e*t al.,* 1999). In Jacobsen syndrome (deletion 11q23),molecular investigations have shown that the terminal 11q23 deletions cluster in a defined region in most patients (Jones *et al*., 1994; Tunnacliffe et *al*., 1999; Jones *et al*., 2000).

Breakage of fragile site can cause terminal deletions in chromosomes (Jones *et al*., 1994; Jones *et al*., 1995). In Jacobsen syndrome, the 11q23 region contains the proto-oncogene *CBL2*, which also contains a CCG trinucleotide repeat (Jones *et al*., 1994; Jones *et al*., 1995; Jones *et al*., 2000). Expansion of this repeat may result in the folate-sensitive fragile site *FRA11B* that is potentially the site of breakage for terminal deletions of 11q23.

Analysis on five Jacobsen syndrome patients and their parents found two cases of deletion to be derived from a *FRA11B*-expressing chromosome (Jones *et al*., 1995; Jones *et al*., 2000).
However, for the remaining three families investigated, the site of breakage was proximal to *FRA11B* (Jones *et al*., 1995).

Terminal deletion can also be due to double-strand DNA breaks of unknown causefollowed by the addition of the telomeric sequence (TTAGGG)n as described in an αthalassemiamutation

associated with terminal truncation of chromosome 16p13.3 (Wilkie *et al*., 1990; Lamb*et al*., 1993; Flint *et al*., 1994). Another characteristic of terminal deletions may be a preferencefor the maternal or paternal chromosome, and it was foundthat in deletion 1p36, 78% of the cases involve a deletion on the maternally inherited chromosome (Wu *et al.*, 1999).

2.2.2.3.2 Interstitial deletions

Several genetic syndromes have been recognized to be caused by interstitial deletions and in majority of the patients, the deletion size is similar (Greenberg *et al*., 1991; Guzzetta *et al*., 1992; Mutirangura *et al*., 1993; Juyal *et al*., 1996; Carlson *et al*., 1997; Chen *et al*., 1997; Wu *et al*., 1999).

The common deletion in 7q11.23 found in Williams syndrome is about 1.6 Mb in size (Peoples *et al*., 2000) and is present in more than 99% of patients (Morris and Mervis, 2000). The deletion size in most patients with Prader-Willi syndrome or Angelman syndrome is about 4 Mb (Mutirangura *et al*., 1993). However, two different proximal breakpoints have been identified in both the maternally derived deletions of Angelman syndrome and the paternally derived deletions of Prader-Willi syndrome (Christian *et al*., 1995). The common Smith-Magenis syndrome deletion within 17p11.2 is approximately 5 Mb (Trask *et al*., 1996; Chen *et al*., 1997) and is found in most patients (Juyal *et al.*, 1996; Chen *et al*., 1997). For most patients with DiGeorge syndrome/VCFS, the deletion in 22q11.2 is about 3Mb (Morrow *et al*., 1995; Carlson *et al*., 1997). Some patients have an alternate distal deletion breakpoint, resulting in a smaller, 1.5-Mb deletion (Carlson *et al*., 1997). Although altered sized deletions, or even rarer unique deletions, can be found in patients with these syndromes, the finding of the same-sized deletions

in most patients points to a specific mechanism giving rise to most of these structural rearrangements.

2.2.2.4 Duplications

Crossing over between nonallelic, directly repeated, homologous segments between sister chromatids (intrachromosomal) or between homologous chromosomes (interchromosomal) would beexpected to produce two reciprocal products: a tandem or direct duplication and a deletion. Duplication in 17p12 results Charcot-Marie tooth disease type 1A (CMT1A) while Smith Magenis syndrome is cause by the corresponding deletion. Homologous recombination between 24-kb flanking repeats, termed CMT1A−REPs, results in a 1.5-Mb deletion that is associated with hereditary neuropathy with liability topressure palsy (HNPP) and the reciprocal duplication product is associated with CMT1A (Lupski, 1998). del (17) (11.2) causes Smith-Magenis syndrome (SMS) and this sameregion was identified to be duplicated in seven unrelated patients (Potocki *et al*., 2000).

2.2.2.5Inversions and insertions

An inversion involves two breaks in a chromosome and the segment is reversed or inverted in the position. If the inversion is outside the centromere, it is termed a paracentricinversion whereas inversion spanning the centromere, involving both the chromosomearms, is known as pericentric inversion. Since it is a balanced rearrangement, it usuallyhas no adverse effect on the carriers unless one of the breakpoints disrupts an importantgene. Pericentric inversion of chromosome 9 is an example of a common structural variantor polymorphism and is not thought to be of any functional importance. A study of377,357 amniocentesis estimated the rate of

inversions to be 1 in 10,000 with a 9.4% riskof an associated congenital abnormality (Warburton, 1991).

An insertion occurs when a segment of a chromosome is inserted in another chromosome.If the inserted material has moved from another chromosome then the karyotype isbalanced. Otherwise it causes an unbalanced chromosome to complement. It can behazardous if it involves the coding region of a gene (Warburton, 1991).

2.2.3 Mosaic trisomy 21

It is a free trisomy 21 but only some cells have an extra chromosome 21.Mosaicism is defined as having two or more genetically distinct cell lines. Approximately 2-4% of Down syndrome patients are mosaics (Aula *et al*., 1973; Mutton *et al.*, 1996; Nguyen and Riess, 2009; Papavassiliou *et al*., 2009).

Mosaicism is a condition in which different cells within an individual have a different genetic makeup. Mosaicism of trisomy 21 occurs due to a mitotic error after fertilization occurs. Cells start out karyotypically normal after fertilization. At some point during cell division, differentiation, and growth a mitotic error occurs for a dividing cell (Peterson and Mikkelsen, 2000). That particular cell then has 47 chromosomes instead of 46. All cells that are copied from this cell during future rounds of mitosis also have 47 chromosomes. This results in a mixture of aneuploid and euploid cells, which make the individual mosaic for trisomy 21 (Freeman *et al*., 2007). Affected individuals have trisomic (47, XX, +21 or 47, XY, +21) and euploid (46, XX or 46, XY) cell lines (Papavassiliou *et al*., 2009). The timing of the occurrence of this error has a

significant effect of phenotypic outcome. For instance, if the error occurs early on it will affect many more cell lines than if it were to occur further along during development after various cell populations have differentiated. This series of events ultimately produces a mosaic effect on the phenotype because some cell populations exhibit trisomy 21 whereas other cell lines are karyotypically normal. The proportion of trisomic cells may vary by cell type and embryological origin (Papavassiliou *et al*., 2009). For instance, karyotype analysis from buccal mucosa and lymphocytes may yield significantly different percentages of trisomic cells and in one instance an 11-yr. old with mosaic DS was 50% trisomic based on buccal cell counts and 7% trisomic based on lymphocyte cell counts (Papavassiliou *et al*., 2009). Analyses based on buccal mucosa and lymphocytes are currently based on 500 or 1000 cell counts (Papavassiliou *et al*., 2009), but in older studies as few as 10 cells may have been analyzed to evaluate the presence or absence of trisomy 21. Moreover, rather than examining all cells collected, in some instances conclusions from these analyses may have been based upon examination of only the first few cells. In other words, an individual may have been diagnosed with full DS (from nondisjunction) or as not having DS based upon examination for 2-3 cells out of the 10 collected, which heightens the likelihood that mosaic DS has been misdiagnosed and underdiagnosed in the past.

Individuals with mosaic trisomy 21 frequently have milder phenotypic differences, lower cognitive impairment (as measured by IQ), and tend to reach developmental milestones sooner than individuals with full trisomy 21 (Fishler *et al*., 1976; Papavassiliou *et al*., 2009). The 1- and 5-year survival rates of children diagnosed with mosaic DS are 97.5% and 95.7% respectively, which is higher than the corresponding non-mosaic survival rates of 92.9% and 88.6% respectively (Rasmussen *et al*., 2006; Shin *et al*., 2010). Children with mosaic DS have a

significantly lower prevalence of major congenital heart defects requiring surgery (36.4%) compared to children with non-mosaic DS (49.3%) (Papavassiliou *et al*., 2009; Shin *et al*., 2010). Mosaic DS accounts for some estimated 1-4% of individuals diagnosed with DS (Peterson and Mikkelsen, 2000; Pueschel 2001; Kava *et al*., 2004; Papavassiliou *et al*., 2009; Shin *et al*., 2010).

2.3 Clinical Diagnosis of Down syndrome

Dr. Langdon Down (1828-1896) was the first to describe the clinical features of Down syndrome children precisely (Lejeune *et al*., 1959; Ward, 1999). The knowledge of clinical manifestations of Down syndrome by physicians and other health professionals is important for an early diagnosis to reduce morbidity and mortality of these children (e.g. early operation of heart defects). Furthermore, proper clinical diagnosis of Down syndrome children is important to avoid normal children being investigated for Down syndrome based on only few clinical features (Devlin and Morrison, 2004). In 1976, Jackson *et al* (1976)created a checklist of 25 signs of Down syndrome to predict the presence of trisomy 21 in 291 patients suspected with DS (Jackson *et al*., 1976; Keppler-Noreuil*et al*., 2002).

The most common characteristic features of Down syndrome are facial features, development delay, hearing and visual abnormalities, gastrointestinal anomalies, congenital heart defects, and leukemia particularly acute megakaryoblastic leukemia. As Down syndrome is associated with many congenital abnormalities and health problems molecular mapping of the so called Down-critical region, DCR, of chromosome 21 was undertaken. The mapping provided evidence that the DCRwhich spans 0.4 to 3 Mb on 21q22.2 is playing a role in pathogenesis of Down syndrome (Delabar*et al*., 1993; Sinet*et al*., 1994). This interval is thought to be responsible for

the expression of 13 features contributing to mental retardation, short stature, muscular hypotonia, joint hyper-flexibility and nine morphological signs: flat nasal bridge, protruding tongue, highly arched palate, narrow palate, folded ears, short and broad hands, incurved 5th finger, high Cummins index and gap between 1st and 2nd toes (Sinet*et al*., 1994). The locus D21S55-MX1 which is located in band 21q22.3 is thought to be responsible for the expression ofother six morphological features: epicanthus, oblique eye fissure, brushfield spots, transverse palmer crease, short stature and hypotonia (Sinet *et al*., 1994).

In addition, Down syndrome is associated with many complex clinical features which might be located outside the critical regionof chromosome 21 indicating that more than one region is responsible for the pathogenesis of theDown syndrome phenotypes (Delabar, *et al*., 1993; Sinet, *et al*., 1994). With respect to the clinical features, it is important to emphasize that there is a great variabilityof the frequencies of phenotypic features in individual DS patients.

2.3.1 Leukemia

The association between DS and leukemia was recognized since 1930 (Mejia-Arangure *et al*., 2005).Children with DS have a 10-20-fold increased incidence of leukemia from newborn period to adulthood and a lower but also increased incidence of solid tumors at all ages (Fong and Brodeur, 1987; Boker*et al*., 2001; Hasle, 2001). The most common form of leukemiaduring childhood is acute lymphoblastic leukemia (ALL) with subtype acutemegakaryoblasticleukemia (AMKL) and a subtype of acute myeloid leukemia (AML) (Hitzlerand Zipursky,2005).The mechanism which leads to the increased risk of leukemia in Down syndrome is not known, but

there are several oncogenes which were identified on the long arm chromosome 21 (Sacchi, 1992; Boker *et al*., 2001).

2.3.2. Alzheimer's disease

Down syndrome is associated with early onset of Alzheimer's disease. A study from (St George-Hyslop *et al.,* 1987) suggested that there must be a gene on chromosome 21 involved in Alzheimer disease.

2.3.3. Congenital heart disease

There is a high frequency of congenital heart disease in children with DS ranging between40-60% (Marino, 1993). The most frequent cardiac anomalies seen in DS patients areatrioventricular septal defects (AVSD) (Freeman*et al*., 2009) which affect the mortalityrate of Down syndrome. Other congenital heart diseases are patent ductus arteriosus, interventricularcommunication, tetralogy of fallot, and valve insufficiency. Many Down syndrome children present withmore than one type of congenital heart disease.

2.3.3.1 Atrioventricular septal defect (AVSD)

The term AVSD covers a broad spectrum of CHD characterized by a common atrioventricular junction with coexisting deficiency in the atrioventricular septum. AVSD comprises around 7% of all CHD and is also referred to as an endocardial cushion defect (Calkoen*et al.,* 2016). The common atrioventricular junction is usually ovoid with unwedging of the left ventricular outflow tract from the usual position between mitral and tricuspid valves. Instead of separate inlet valves, the AV junction is guarded by a common valve, which often is comprised of five leaflets, two of which are bridging leaflets across the crest of the interventricular septum (Figure 2.2). These are

termed superior and inferior bridging leaflets, respectively. There is also a left lateral (mural) leaflet, right anterosuperior leaflet, and a right inferior leaflet (Craig, 2006; Calkoen*et al.,* 2016).

The Rastelli classification from 1966 divides complete AVSD into three subgroups on the basis of the anatomy of the superior bridging leaflet and its chordal attachments (Figure 2.3). In Rastelli type A, the superior bridging leaflet is divided at the level of the ventricular septum; in Rastelli type B, the division of the superior bridging leaflet occurs to a right ventricular papillary muscle; and in Rastelli type C, the superior bridging leaflet is undivided or free floating. Rastelli type C is the most common arrangement found in Down syndrome (Cohens, 2009). In complete AVSD, shunting occurs at both atrial and ventricular levels; however, attachment of the bridging leaflets to the crest of ventricular septum results in an exclusively atrial shunt through a primum ASD, also called a partial AVSD, whereas attachment of the bridging leaflet to the atrial septum results in exclusively ventricular shunting (Figure 2.4). Other congenital heart defects commonly associated with AVSD include left ventricular outflow tract obstruction especially in the setting of a Rastelli type A superior bridging leaflet as there is extreme unwedging of the aorta from its usual position and consequent elongation of the outflow tract. Ventricular hypoplasia and atrial isomerism are also described although infrequently with Down syndrome. Tetralogy of Fallot is the most commonly observed association and is seen in up to 6.7% cases of AVSD (Cohen, 2009). There is a high incidence of associated other extra cardiac abnormalities. One study of 87 patients with Tetralogy and AVSD reported that 67% of these patients had Down syndrome (Vergara*et al*., 2006).

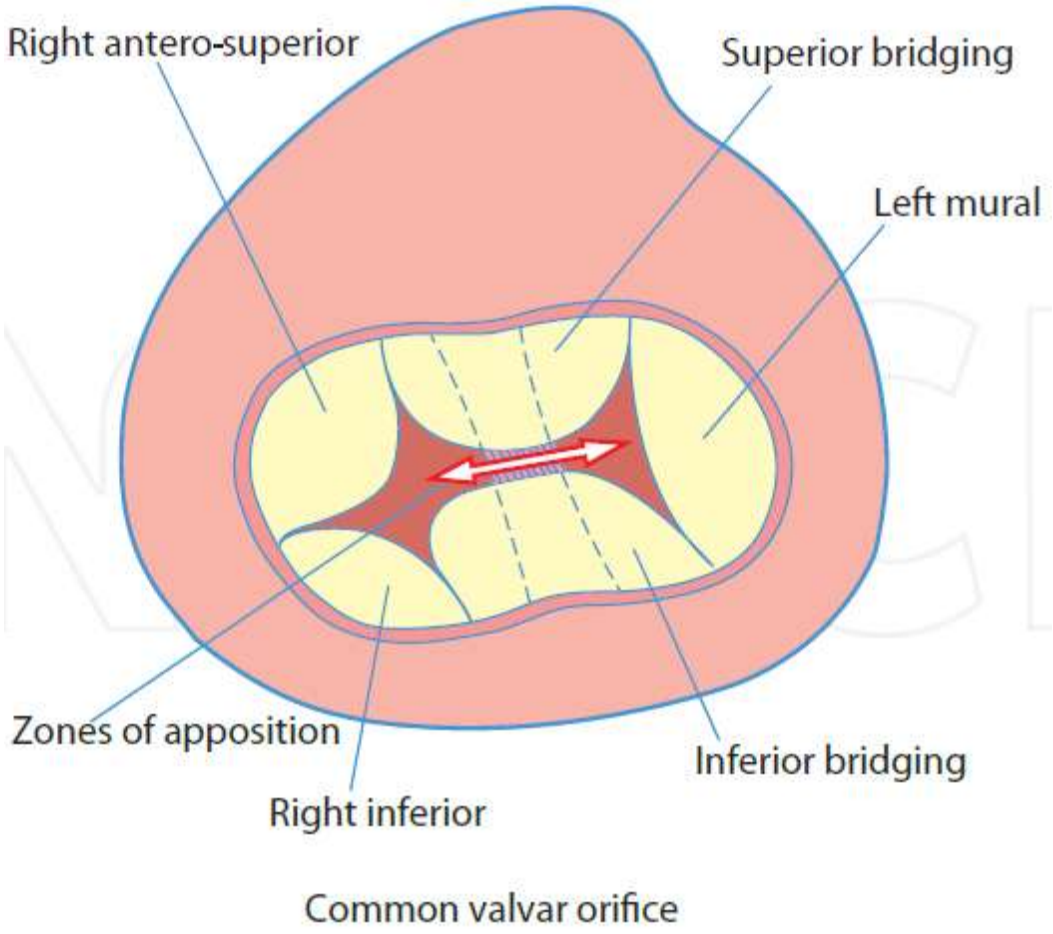


Figure 2.2. The arrangement of the common atrioventricular valve leaflets in complete AVSD(McInnes and Willard, 2001).

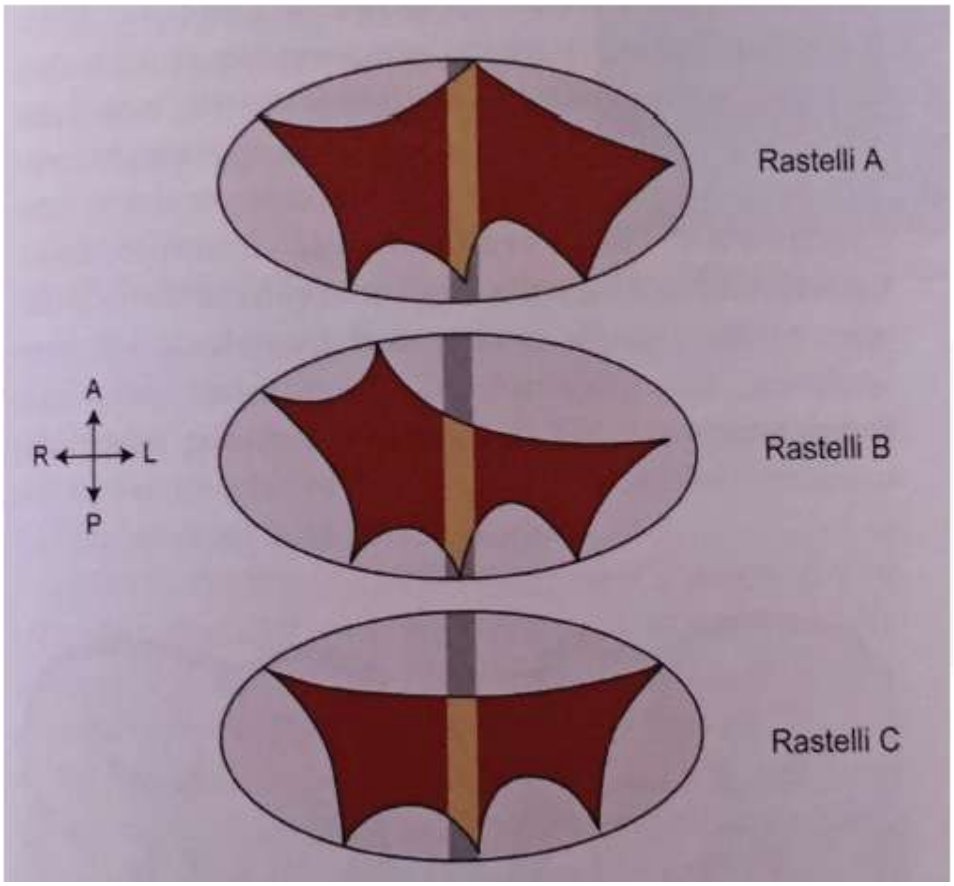


Figure 2.3. Rastelli classification. Type a (top): The superior bridging leaflet is divided at the ventricular septum. Type B (middle): The division occurs to a right ventricular papillary muscle. Type C (bottom): The superior bridging leaflet is undivided (McInnes and Willard, 2001).

2.3.3.2. Primum atrial septal defect/partial AVS

In an isolated primum ASD or partial AVSD, the AV junction is a common structure; however, there are separate right and left AV valve orifices as a band of valve tissue joins the superior and

inferior bridging leaflets. The AV valves appear at the same level, and there may be regurgitation through the zone of opposition or “cleft” in the left AV valve (Figure 2.4). Timing of surgery in this case is less crucial especially if there is minimal AV valve regurgitation. Repair is often carried out in late infancy or early childhood. Isolated primum ASD unrepaired carries 50% mortality before the age of 20 years (Craig, 2006). Surgical results are good, and 30-day and 1-year survival are 98.8 and 98.7%, respectively (NICOR, 2017). Long-term complications are similar to those described following AVSD repair with the most common reason for reoperation being left AV valve regurgitation followed by left ventricular outflow tract obstruction (Najim*et al*., 1997).

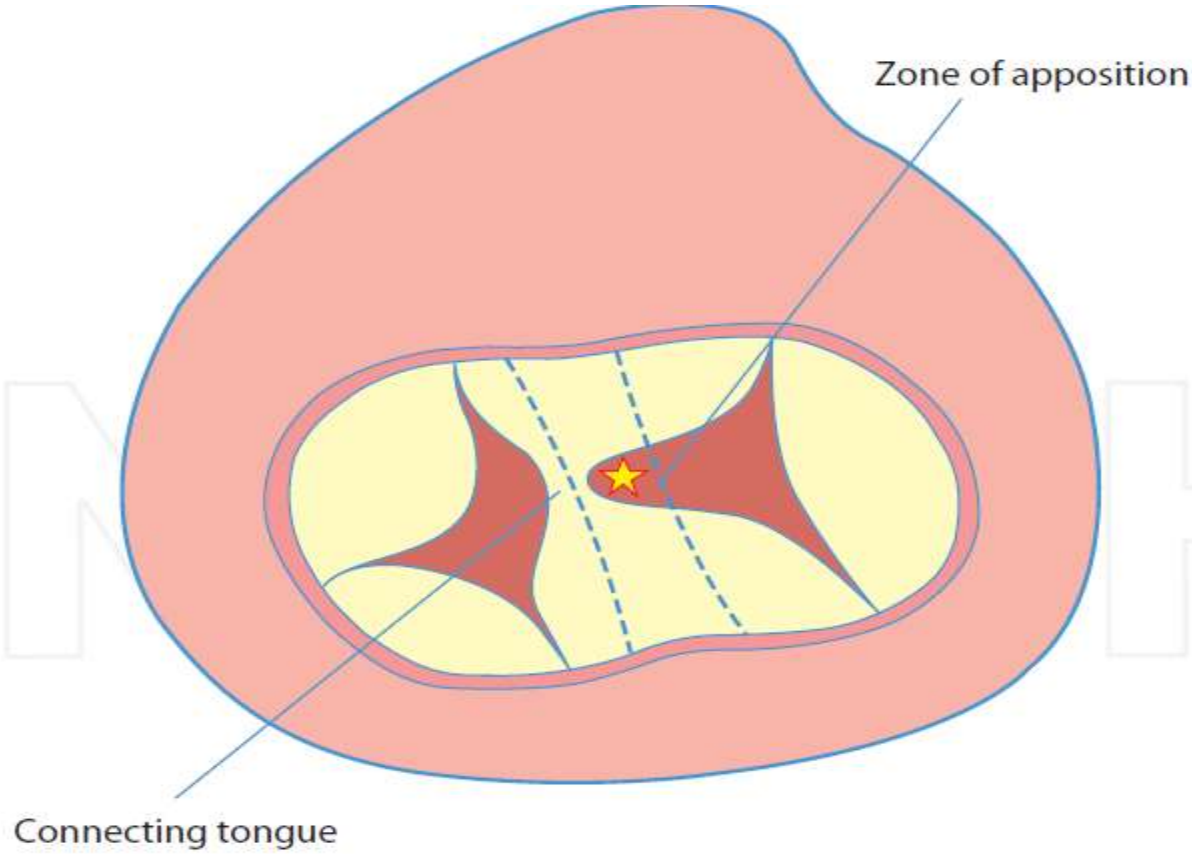


Figure 2.4. Arrangement of the common atrioventricular valve leaflets in primum ASD (McInnes and Willard, 2001).

2.3.3.3.Tetralogy of Fallot

Tetralogy of Fallot is a conotruncal defect caused by the anterior and cephalad deviation of the infundibular septum, which leads to the development of the four characteristic components:

ventricular septal defect, overriding aorta, right ventricular outflow tract obstruction, and right ventricular hypertrophy (Figure 2.5). Tetralogy of Fallot occurs in around 6% of patients with DS and is the most common cyanotic heart defect to present in this patient group. Conversely around 8% of patients with Tetralogy of Fallot have DS, although this is slightly higher in fetal series (Srivastava*et al*., 2009).

Clinical presentation of tetralogy of fallot depends very much on the degree of outflow tract obstruction present. Patients may present with profound central cyanosis in the neonatal period if the obstruction is severe and may actually be duct dependent, i.e., there is insufficient pulmonary blood flow once the ductus arteriosus closes. These patients require palliation with a Blalock-Taussig shunt or ductal stent to secure pulmonary blood flow and permit growth for corrective surgery. If there is little outflow tract obstruction, the patient may exhibit signs and symptoms of congestive cardiac failure as there will be a large left to right shunt through the VSD; in this case there will be little or no cyanosis.

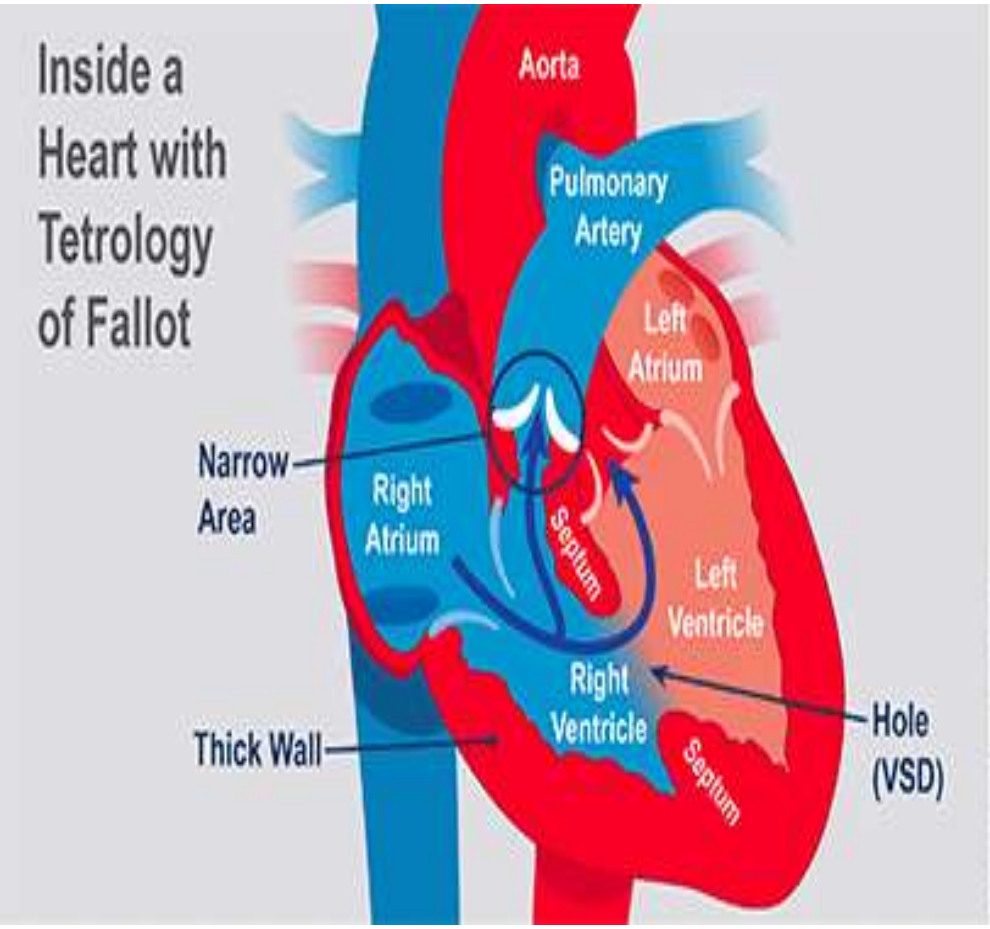


Figure 2.5. Diagrammatic representation of tetralogy of Fallot (McInnes and Willard, 2001).

2.3.3.4. Ventricular septal defect (VSD)

A ventricular septal defect is defined as a hole between the right and left ventricles. In most series it is the second most common form of CHD described in DS. VSDs are generally

classified depending on what portion of the ventricular septum they span, illustrated in (Figure 2.6). In Down syndrome VSDs often occur in the inlet septum (Forbus*et al*., 2009). In a large series of patients with Down syndrome, inlet VSD was one of the most frequently reported subtypes. Muscular and subarterial VSDs were not described (Marino*et al*., 1990). Inlet VSD is associated with abnormalities of the left AV valve with straddling chordal and papillary muscle attachment (Marino*et al*., 1990). In the setting of Down syndrome, these defects likely form part of the AVSD complex described earlier (Marino*et al*., 1990).

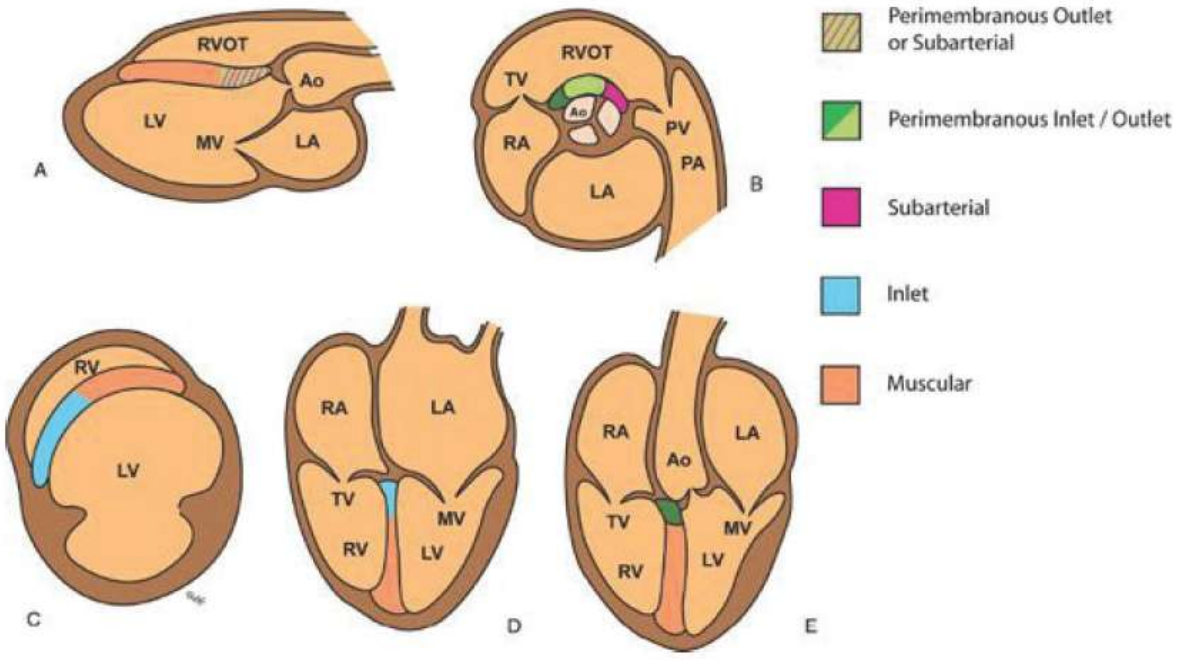


Figure 2.6. Diagrammatic representation of possible VSD locations on standard echo views. (A) long axis view, (B) short axis view at aortic valve level, (C) short axis view through ventricles, (D) four chamber view, (E) left ventricular outflow tract view.

2.4 Genetic Basis of Down syndrome

In Down syndrome, approximately 95% of the cases are due to non-disjunction resulting in an extra copy of a chromosome 21 (trisomy 21) (Lejeune*et al*., 1959). The remaining are due to

translocations involvingchromosome 21 and somatic mosaicism (Sherman*et al*., 2005). Most trisomy 21 cases are due to an error in maternal meiosis, whereby about 70% originate during maternal meiosis I (MI) and about 20% during maternal meiosis II (MII), defective paternal meiosis is found for upto 8-10% of all cases (Savage*et al*., 1998; Petersen and Mikkelsen, 2000; Sherman*et al*., 2005). Even though significant progress has been made in recent years, the causes of the increased non-disjunction rate resulting in trisomy 21 are far from understood. Maternal age, germ line mosaicism, and altered recombination remain the only well-established risk factors for non-disjunction of chromosome 21 (Sherman*et al*., 2005).

In contrast to humans, where up to 50% of all conceptions are aneuploid, non-disjunction in mostmodel organisms are a rare event. For example, in *Saccharomyces cerevisiae* chromosomemalsegregation is estimated to take place every 10,000 meiotic events (Sears*et al*., 1992). For recombination events in *Drosophila malanogaster* oocytes, it has been demonstrated that recombination on MI non-disjunction takes place at the distal part of the chromosome, whilefor MII non-disjunction, it takes place in proximal location (Koehler*et al*., 1996).

2.5 Risk Factors for Trisomy 21

2.5.1 Advanced maternal age

Advanced maternal age at the time of conception is the most established significant risk factorfor meiotic non-disjunction of chromosome 21 (Sherman *et al*., 1994; Sherman*et al*., 2005; Oliver *et al*., 2008). Penrose, 1933, was the first who noted the effect of advanced maternal age on the rate

of DS (Penrose, 2009). About 2% of recognized pregnancies of women under the age of 25 years are trisomic, this increases to 10% for womenof 36 years and to 33% by the age of 42 years (Hassold and Sherman, 2000). The influence ofmaternal age has been observed in all population studies in respect to race, geography orsocioeconomic factors. However, the basis for the effect of increasing maternal age on the non-disjunctionrate is largely unclear. In human female, meiosis starts in the 3rd month of fetal life and is arrested in prophase of MI from 6 months of fetal life onwards until ovulation which takes around 10 to 40 years (Sperling *et al*., 1991; Hassold and Sherman, 2000;; Warburton, 2005,). At the time of ovulation, the oocytes complete MI and progress to MII where they remain arrested until they are fertilized and subsequently complete the meiotic stage MII. Warburton (2005) presented two hypotheses for the effect of maternal age on the non-disjunction rate: the first is that differentvariables which affect the oocytes overtime such as decreased expression of checkpoint proteins which maintain sister chromatid adhesion or meiotic checkpoint, accumulate with increased maternal age resulting in an increased non-disjunction rate (Jeffreys*et al*., 2003; Vogt *et al*., 2008).

A second hypothesis is that biological aging of the oocytes is animportant factor and that the frequency of trisomic conceptions will depend upon the biological age of the women's oocytes, rather than upon the chronological age (Warbuton, 2005).

2.5.2. Maternal recombination

Altered recombination is another important factor after maternal age which is associated with non-disjunction error. Warren *et al*., 1987 were the first who provided evidence that a

proportionof maternal non-disjunction errors were associated with reduced recombination along chromosome 21. Further studies (Antonarakis *et al*., 1992; Antonarakis *et al*., 1993; Sherman *et al*., 1994; Yoon*et al*., 1996; Sherman*et al*., 2005)regarding the etiology of Down syndrome demonstrated a relationship between the non-disjunction event and altered recombination. Most of these studies approved that the location of the recombination is a risk factor for non-disjunction of trisomy 21 (Yoon*et al*., 1996; Savage*et al*., 1998; Sherman*et al*., 2005; Oliver*etal*., 2008).

Concerning the location of the recombination associated with non-disjunction, three susceptible exchange patterns have been demonstrated for maternal non-disjoining error:

(1) No exchange leads to an increased risk of MI error

(2) A single telomeric exchange leads to increased risk ofMI error, and finally

(3) A pericentromeric exchange leads to increased risk of MII error (Hassoldand Sherman, 2000; Sherman*et al*., 2005; Oliver*et al.*, 2008).

The association of maternal MII errors with specific recombination pattern is thought to be initiated in MI, at least for a certain proportion of MII errors (Sherman *et al*., 2005). A study in the USA population aimed to examine the number and location of recombination by age group (Oliver *et al*., 2008). The results suggested that the risk imposed by the absence of exchange or by a single telomeric exchange is the same irrespective of the age of the oocyte, while the riskimposed by a single pericentromeric exchange increases with increasing maternal age. Oliver'sfindings were supported by a study from an Indian population, where the author

suggested that the genetic etiology underlying the occurrence of trisomy 21 may be similar across human populations (Ghosh*et al*., 2009).

2.5.3 Paternal risk factor for chromosome 21 nondisjunction

The paternal error constitutes nearly 5 to 10% of total occurrence of live born DS cases, dependingupon the populations studied. Unlike maternal cases the studies on the etiology ofpaternal NDJ are limited by insufficient sample size. The first significant report was providedby Savage *et al*., (1998) who found reduction in recombination in MI non-disjoined cases, but notin MII errors.

Moreover, the authors inferred that altered chiasma positioning maynot associate with NDJ in spermatogenesis, as the authors recorded very concordant patternof chiasma distribution among DS cases and control. In their extension study with morepaternallyderived samples, Oliver *et al*., (2009) determined that majority of Ch21 NDJ errors inspermatogenesis occurs at MII (32%MI:68%MII), and the authors did not found significantreduction in recombination either in MI or in MII errors. Moreover, their sample did not exhibitany advanced age effect for either of meiotic outcome groups. The authors argued thatthe time scale of spermatogenesis is much shorter starting at puberty runs continuouslywithout meiotic halt and this explains why advancing paternal age does not exacerbate andassociate Ch21 NDJ in spermatogenesis. This study is significant in the realization that etiologyof Ch21 NDJ differs in two sexes and case of paternal errors remains an enigma. In general,the frequency of recombination for normally segregating chromosome is less in malethan in female. But further reduction in recombination

frequency may not cause NDJ inmale. Moreover, epidemiological study on the risk factors for paternal NDJ of Ch21 is yet to be conducted.

2.5.4Abnormal folate and methyl metabolism in mothers with Down syndrome

There are some studies which indicate that alterations in the folate metabolism are risk factors for trisomy 21 (Hobbs*et al*., 2000; James, 2004; Takamura *et al*., 2004; Eskes, 2006;Rai*et al*., 2006).Genes involved in the maternal folate metabolism have been hypothesized to be candidate genes involved in an elevated non-disjunction rate. It has been shown that the 677C T polymorphism in the methylenetetrahydrofolate reductase (*MTHFR*) gene increased the risk of having a child with Down syndrome (OR = 2.6)(James, 2004). *MTHFR* catalyzing the conversion of 5, 10-methylenetetrahydrofolate, the methyl donor for the remethylation of homocysteine to methionine. Mutation of the *MTHFR* gene (677C→T) causes the substation of alanine to valine in the *MTHFR* protein and reduces enzyme activity. Activity of *MTHFR* is reduced to 37% for heterozygous *C/T* genotype, and 70% with homozygous *T/T* genotype in relative to normal *C/C* genotype (James, 2004). The authors assume that low folate status, whether due to dietary or genetic factors, could induce centromeric DNA hypomethylation and alterations in chromatin structure which adversely affect DNA-protein interactions required for centromericcohesion and normal meiotic segregation. However, various other studies could not confirm these results. It was suggested that one possible explanation for the inconsistent results among the numerous studies may reflect the complex interaction between effects of genetic variants and nutritional intake (James, 2004).

2.5.5. Parentalgermline mosaicism

Parental gonadal mosaicism has been suggested by many studies as a risk factor for cases in families with multiple trisomy 21 conceptions (Tseng *et al*., 1994; Nielsen*et al*., 1988; Cozzi *et al*., 1999; Bruyere*et al*., 2000). If parental gonadal mosaic is present the recurrence risk will be higher and will depend on the proportion of trisomy 21 cells present in the gonads. Therefore, in families with one affected child with free trisomy 21, it is assumed that the recurrence risk estimates to 1-2% based on live births and prenatal diagnosis (Nielsen*et al*., 1988; Bruyere*et al*., 2000). Studies of genetic implantationdiagnosis indicate that aneuploidy in oocytes and embryos is not a rare event and that it increases with maternal age because of trisomic germ line and disruption in meiotic division (Munne*et al*., 1994).

2.5.6. Mutations in nuclear encoded genes

The mechanisms of meiosis reveal three specific processes: (1) pairing and synapsis of homologous chromosomes, (2) reciprocal meiotic recombination (crossover) and (3) regulationof sister chromatid cohesion(SCC) (Matsuura *et al*., 2000; Champion and Hawley, 2002;Nasmyth, 2002). Somemutations which control the above-mentioned processes may lead to a defect in chromosome segregation and produce cells that are aneuploid. Some studies reported certain gene mutations in model organisms such as Drosophila, Sacchromyces cerevisiae, and mice (Rockmill and Roeder, 1990; Knowles and Hawley, 1991; Baudat*et al*., 2000; Halverson*etal*., 2000). In humans, several mutations in genes implicated in chromosome segregation have been

identified, that increase the risk of mitotic non-disjunction in somatic cells such as the MAD2 and BUB1 gene (Table 2.1).

Table 2.1: Selection of genes affecting mitotic non-disjunction in man

Germline mutations	Somatic Mutations
• Apple-Peel syndrome (OMIM 243605)•	defective MAD2 gene (OMIM 601467)
• Mosaic variegated aneuploidy syndrome •	defective BUB1 gene (OMIM 602452)
• MVA with total premature chromatid separation (OMIM 176430)	
• Roberts syndrome (OMIM 268300)	
• RECQ4-deficiency (Rothmund- Thomson	

S; OMIM 268400)

2.5.7. Mitochondrial (mtDNA) mutations

It has been hypothesized that mtDNA mutations may play a role in the etiology of Down syndrome. Thenumber of mitochondrial mutations increases with age in different cells specifically in oocytes (Arbuzov*et al*., 2002). The authors suggested as a possible explanation that mutationsin mtDNA may reduce ATP levels and increase the generation of free radicals, which could inturn affect the synaptonemal complex formation, chromosome segregation, the division spindle,and alter recombination (the enzymes participating in recombination and DNA repair are ATPdependent) leading to aneuploidy (Arbuzova*et al*., 2001; Arbuzova*et al*., 2002).

2.5.8. Consanguinity

Consanguineous marriages are traditionally common among Arab countries. This leads to an increased birth prevalence of infants with recessive diseases, congenital anomalies, morbidity and mortality. The Omani society has, as other Arab countries, a long tradition of consanguinity. Rajab and Patton reported that among 60,635 Omani couples 24.1% were marriages between firstcousins, 11.8% between second cousins, and 20.4% were within specific tribal groups (Rajab and Patton, 2000). Individuals who are closely consanguineous have a higher probability of carrying rare recessive alleles which can be transmitted homozygous to their offspring's.

Consequently, it is conceivable that homozygous gene mutations, in a gene influencing nondisjunction, could result in an increased aneuploidy rate of the progeny. Thus, it cannot be excluded that an increased non-disjunction rate could result from a recessive gene in combination with other risk factors, specifically in younger aged mothers. There are some publications which report on a positive association between Down syndrome and consanguinity and the possible involvement of recessive genes in non-disjunction (Alfi*et al*., 1980; Farag and Teebi, 1988). Such observations have been made in Shetland (Roberts*et al*., 1991) and Canada (De Braekeleer and Dao, 1994) and are explained by recessive genes, possibly preventingthe loss of the trisomy 21 fetus. Some other data did not support the association betweenconsanguinity and Down syndrome. Basaran *et al*,(1992) reported a lower consanguinity rate and inbreeding coefficient among parents of Down syndrome than in parents without DS children (Basaran*et al*., 1992). Similar findings were reported from Kuwait demonstrating that the frequency of consanguineous marriages among controls was higher than that among Down syndrome families,

though Kuwait has a highly inbred population with 54.3% of consanguineous marriages (Al-Awadi*et al*., 1985).

2.5.9. Exogenous risk factors

There is increasing evidence that maternal meiosis is an error prone process that is most sensitive to the effect of exogenous factors at the time of chromosomal segregation, which is around conception. This is supported by two convincing associations in which two local clusters of trisomy 21 were explained by hazards occurring around the time of conception: the ingestion of a chemical, trichloroform, employed against fish parasites (Czeizel*et al*., 1993) and the inhalation of iodine-131 from the Chernobyl reactor accident (Sperling *et al*., 1994).

Already in 1976 it has been shown that the Down syndrome prevalence is increased in certain regions in Kerala with high background radiation (Kochupillai*et al*., 1976). Another two studies, one conducted by EUROCAT, demonstrated a higher risk of chromosomal anomalies in people who lived close to hazardous waste landfill sites (0-3 km) than in those who lived further away (3-7 km). The EUROCAT study investigated 245 cases of chromosomal anomalies and 2412 controls who lived near 23 such sites in Europe (odds ratio 1.41, 95% CI 1.00-1.99)(Geschwind, *et al*., 1992; Vrijheid*et al*., 2002). Many other exogenous factors such as maternal irradiation, alcohol, fertility drugs, low economic status etc. have been implicated in an increased non-disjunction rate (Boue and Boue, 1973; Harlap*et al*., 1979; Uchida,1979;Kaufman 1983; Strigini *et al*., 1990; Yang*et al*., 1999; Torfs and Christianson, 2003; Christianson*et al*., 2004; Padmanabhan*et al*., 2004). Thus, it seems certain that environmental factors are involved in the etiology of Down syndrome.

There is increasing evidence that maternal meiosis is the main risk for non-disjunction error dueto the lack of checkpoint control during chromosomal segregation (LeMaire-Adkins*et al*., 1997) which makes it conceivable that this process is also sensitive to the effect of endogenous and exogenous factors (Table 2.2) (Sperling*et al*.,1991).

Table 2.2:Represent risk factors of meiotic non-disjunction in man

Increasing maternal age:	**Monogenic risk factors:**
• limited oocyte pool	• defective folate metabolism
• two hit model susceptible bivalent	• apolipoprotein ε4 allele
• abnormal processing of metaphase I	•presenilin-1 gene polymorphism
• defective spindle formation	•impaired function of mitochondria
• defective checkpoint control	•consanguinity
Chromosomal risk factors:	**Environmental risk factors:**
• size of chromosomes	• parental irradiation

• NOR variants	• oral contraceptives
• aberrant centromere structure	• fertility drugs
• premature centromere division	•thyroid antibodies

• viral infection

•Ingestion of metriphonate

Others:

• reproductive activity

• seasonal variation in endocrine factors

2.6. Polymerase Chain Reaction Principle

The polymerase chain reaction (PCR) is considered to be one of the most advanced technologies in the field of molecular biology developed in 1980's by Kary Mullis (Mullis*et al.*, 1986; Mullis 1990). One of the advances of PCR is that only very small amounts of DNA are needed extracted from blood samples, hair roots or tissues. PCR is applied in many research and medical diagnostic fields such as diagnosis of hereditary diseases, identification of infectious diseases and also identification of genetic fingerprints in addition to other applications.

PCR is used to amplify specific fragments of a DNA strand and it is based on the enzymatic amplification of a target DNA sequence flanked by a pair of oligonucleotide primers. By PCR it is possible to amplify a single or few DNA copies to millions of copies of this DNA fragment using 20 to 40 PCR cycles. Each PCR cycle requires 3-steps:

(1) Denaturation of the DNA at high temperature (94-96ºC)

(2) Annealing step (54-65ºC) allowing the primers to hybridize to opposite strands of the target DNA

(3) Elongation (72ºC) or extension of primers by a heat stable DNA polymerase (Taq) which is isolated from thermophilic bacteria.

PCR requires several necessary components and reagents including: DNA template that contains a DNA region to be amplified, a forward and a reverse primer which are complementary to the DNA region at 5´ and 3´ ends of the DNA template, buffer solutions for optimum activity and stability of the DNA polymerase, DNA polymerase such as Taq polymerase or any other head stable DNA polymerase, deoxynucleotide triphosphatase (dNTP's) and finally Mg^{2+}.

2.7. Microsatellite Analysis

Microsatellites, also known as simple sequence repeats (STR), are highly informative DNA sequences in the human genome. They are commonly used for mapping, population studies, linkage analyses and to trace inheritance patterns. STRs are short tandem repeats which are highly polymorphic due to a variation in the number of repeating units between alleles within a population. The short sequences are repeated in tandem arrays and the length of sequences are most often di, tri, or tetra nucleotides, each repeated 5-50 times at a locus(Koreth *et al*., 1996). Most microsatellites occur in non-coding or intronic regions of the genomic DNA.

The length of the microsatellites can be determined by PCR using primers that flank both ends of the microsatellite sequence producing DNA fragments which length depends on the number of repeats in the microsatellite. These fragments are analyzed by using DNA sequencing instrument

utilizing capillary electrophoresis by which the fragment size can be determined. In order to distinguish between fragments varying in length by few bases, the size resolution should be good enough. A size standard is run in each capillary to create a standard curve of sufficient precision. The size standard has to be labelled with a different colored fluorescent dye from the fragment to be analyzed which allows multiplexing of different fragment analysis in each capillary separation run. The microsatellites for diagnosis of trisomies should be polymorphic with a high level of heterozygosity. Furthermore, different STRs were used for chromosome 21 to insure informative results.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Materials used for karyotyping

The following were the reagents and equipments used for the study:

Reagents:RPMI-1640 medium w/25MM HEPES (Gibco);W/L-Glutamine; W/O NaHCO3 11x liquid (Gibco), Foetal Bovine Serum Heat inactivated, KaryoMax®Colcemid®Solution, Potassium Chloride, Glacial Acetic Acid AnalaR®, Methanol AnalaR®, Trypsin 250, Phytohaemagglutinin (PHA), Penicillin, Streptomycin sulphate, Giemsa's stain Powder, Sodium Chloride (NaCl), Glycerol, Ethanol (Spirit), Potassium dihydrogen phosphateMonobasic (KH2PO4), DPX mount media

Equipments: Incubator, 37ºC, Biological safety cabinet class II, Automatic dispenser, Slide warmer, 37ºC, Phase microscope (BX51), Drying oven, 60ºC, Centrifuge, Vortex, Vortex (Vysis) Pasteur pipette, Disposable centrifuge tubes, 15ml, 10ml sterile pipettes, 100 ml measuring cylinders, Disposable syringe (1ml), Heparinized tubes (green), Gloves, Frosted

microscope slides, Cover glasses, 22x50mm, Coplin jars, 100ml, Measuring cylinders, 500ml, Slide holders, Slides storage box, Microil immersion, Water bath.

3.1.2 Materials used for PCR and STR analysis

The following were the reagents and equipments used for the study:

Reagents: D21S11 (primer), dH2O - Primer-Mix –(5.0 µl), PCR Reaction Mix –(9.2 µl) Ampli Taq Gold DNA Polymerase (5U/µl 0.8 µl).

Equipments: Thermocycler with gradient (ABI), Microcentrifuge (Eppendorf),Pipette set with different ranges (10µ1, 20 µ1 and 200 µ1) (Eppendorf), 96 PCR plate Full Skirt (Eppendorf) Sealing tape Covers (Sarstedt) Pipette tips, sterile, disposable hydrophobic filter-plugged (Eppendorf) Vortex (IKA®Labortechnik), Minicentrifuge (Fisher Scientific) Sequencing plate (96 PCR plate half Skirt) (Thermo Scientific) PCR 0.5ml tubes (for master mix preparation) (Eppendorf), Multi-rack (New Lab) Gloves, disposable (Charite)` Microcentrifuge tube rack (New Lab), Genescan analysis software (ABI)

3.1.3 Materials used for DNA extraction

The following were the reagents and equipments used for the study:

EDTA bottle, 10% SDS, isopropanol, Nacl, 10Mm Tris-Hcl, ethanol, distilled water, water bath, ice and proteinase.

3.1.4 Materials used for DNA PAGE Gel electrophoresis

The following were the reagents and equipments used for the study:

Reagents: Acrylamide/bisacrylamide stock solution for DNA gels. Urea. 1× TBE electrophoresis buffer. Deionized water. Ammonium persulfate. Tetramethyl ethylenediamine(TEMED) electrophoresis grade.

Equipment's: Gloves. Safety glasses. Laboratory wipes. Pipette. Ethanol, soap, and glass cleaner,beaker,water bath, clips, spacers and combs.

3.2. Study Location and Subjects

The study was conducted in two tertiary health institutions in Kano metropolis namely: Murtala Muhammad Specialist Hospital and Hasiya Bayero Paediatric Hospital.

Kano State is located between latitude 12.2^0North and longitude 9.4^0 East with the Kano city as the capital of the State (Figure 3.1). The State at present is the most populous in Nigeria, with over 9,000,000 people (NPC/FGN, 2007). Mainly Hausa and Fulani tribes reside in Kano. The urban Fulani have been absorbed into Hausa community through intermarriage over many centuries and as such are being considered as Hausas. Hausas and Fulani constitute about 85% of the population in the city and about 97% in the villages. A clear majority are Muslims by religion. Hausa is the lingua franca, but English is the official language. A greater proportion of the population is engaged in the cash-based occupation (Business). The middle class consists mostly of civil servants while in the villages, most of the people are subsistence farmers (Olofin, 1987). The study was conducted between January 2017 andJuly 2018. The study includes

referrals to the hospitals from various hospitals in the State. The Murtala Muhammad Specialist Hospital (MMSH) was founded in 1924 and has four major clinical departments; Medicine, Surgery, Pediatrics' and Obstetrics and Gynaecology. The Paediatrics department has the following units: neonatal unit (nursery), emergency paediatric unit (EPU), paediatric outpatient unit, diarrhoel training unit (DTU), sickle cell unit, and family care centre (FCC), two wards and side laboratory. The number of patients in paediatric outpatient is seasonal. The department has 60 bed spaces, 18 Neonatal unit, outpatient approximately 200 per day and more than 300 patients during seasonal epidemics.

3.2.1Subjects

Children aged between 0-14 years were seen at the Paediatric clinic with facial features such as mental retardation, hypotonia, congenital heart defect, hypertelorism, who have been diagnosed with DS by the paediatricians.All patients seen with DS until sample size were reached and compared with age- sex matched controls. The controls were also recruited from pediatric clinic, immunization centers and general outpatient's department of Murtala Muhammad Specialist and Hasiya Bayero Pediatric Hospitals.A total of 35 subjects with DS were recruited and 16 among them were sampled for karyotyping and PCR using STR markers.

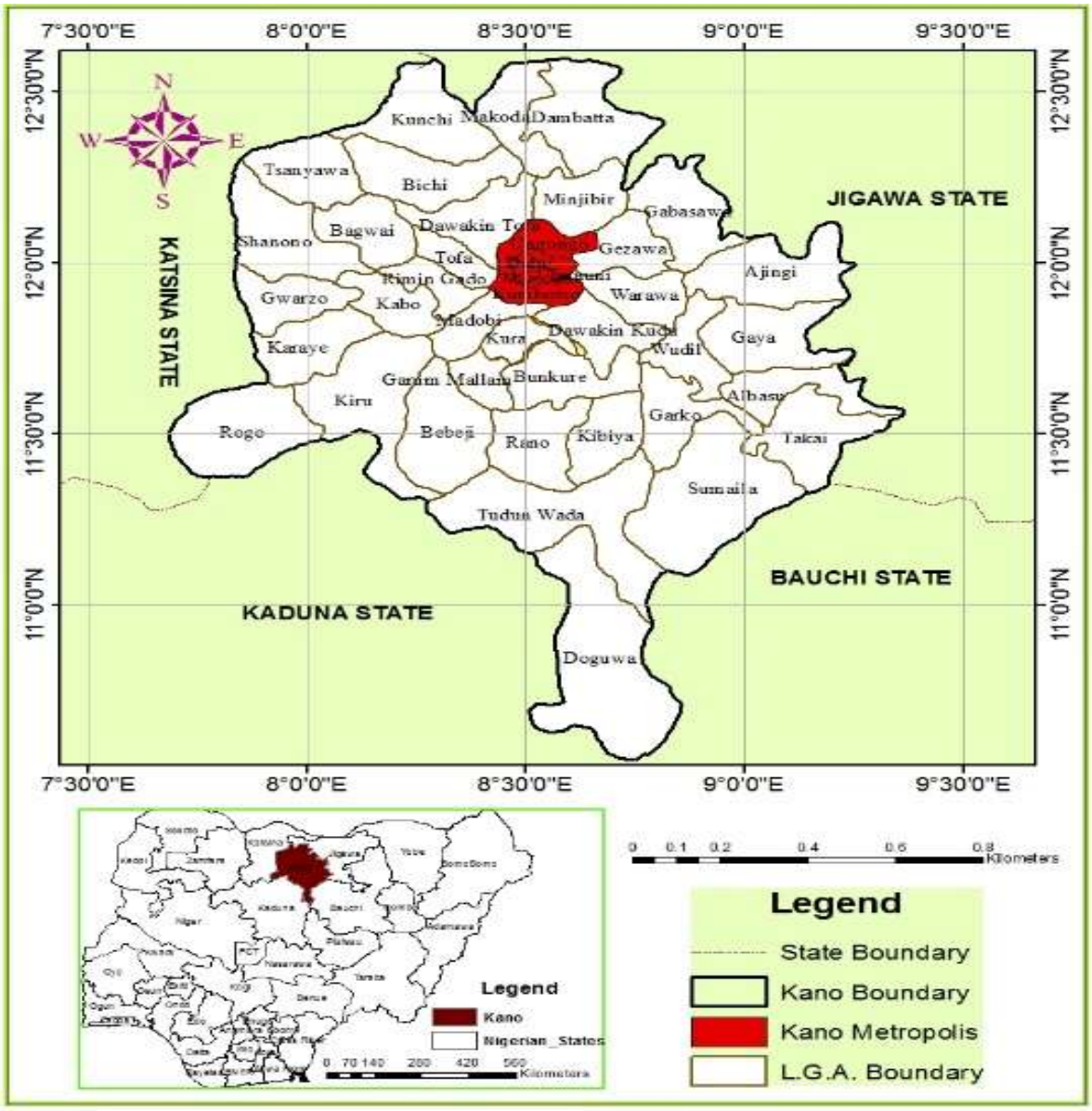


Figure 3.1: Map locating Kano metropolis within Kano State (Olofin, 1987)

3.2.2 Inclusion criteria

The following were used as inclusion criteria

i. Any child clinically diagnosed with Down syndrome attending the Paediatric clinic of any of the two study health facilities.
ii. Any clientwhose caregiver (s)gave his/her consent to participate in the study.
iii. Children whose both parents are Nigerians.

3.2.3 Exclusion criteria

i. Any client whose caregiver (s) do not give their consent.
ii. Subjects with craniofacial deformities or abnormalities not diagnosed as Down syndrome
iii. Patients who have obvious bossing syndrome i.e. sickle cell patients or metabolic conditions like rickets.

3.2.4 Sample size determination

The sample size was determined using the formula below; (Naing, 2006)

$$n = \frac{Z^2pq}{d^2}$$

Where n= desired sample size

Z= standard normal deviation 1.96 at 95% confidence level

q= 1 – p

d= degree of precision

p= prevalence = 1.16%

$$n= \frac{(1.96)^2(0.0116)\ (0.988)}{(0.05)^2}$$

n=18

(Adding 10% non-response rate) 18/0.9= 20

Total sample size= DS (35) +Control (35) =70(age-sex matched)

3.3. Sampling Technique

The study was designed to be a cross sectional observational study. All patients seen with Down syndrome in the facilities until sample size were reached.

3.3.1 Ethical approval

Proposal was submitted to the Ahmadu Bello University Research Ethical Committee and Kano State Hospital Management Board ResearchEthics Committee for evaluation and approval. The following guidelines were observed:

i. Down syndrome children wereseen by the paediatricians.
ii. there was intervention; drugs and invasive procedures in babies and mothers.
iii. procedure wascarriedwiththe consent of the participants or family concerned.

Ethical approval was obtained from the Ethical Committee of Kano Ministry of Health and Kano State Hospital Management Board through the management of Murtala Muhammad Specialist Hospital, Kano.

3.3.2 Informed consent

A written consent was obtained from parents/caregivers of clients for participation after briefing them on the studies. (Appendix 1).

3.3.3. Clinical examination

1. All children had general physical examination and their craniofacial and dermatoglyphic features of the hand were noted and documented for both subjects and control.
2. 2D echocardiographic examinationreports were retrieved from patient's case notes and reports were documented.
3. Biodata and socio demographic data of subjects/control and parents were obtained from questionnaire (Appendix I) and analysed.

3.4 Cytogenetic Analysis

3.4.1 Patients recruitment

Down syndrome patients (n=35) were recruited and 16 among them were sampled for karyotyping from Murtala Mohammed Specialist and Hasiya Bayero Paediatric Hospitals in Kano, Kano State, Nigeria

3.4.1.1 Clinical procedure of obtaining blood from the Down syndrome subjects

The DS patients were identified and selected from the hospital case notes. From each subject, 5ml of venous blood sample was collected using a sterile 21G needle fitted with syringe. Blood collection was done during the morning hours to avoid the effect of diurnal variation or circadian rhythm in the blood which are parameters to be measured. Standard technique of venipuncture and universal safety precaution was employed. Blood sample was transferred into a heparinized

bottle and allowed to stand. The blood samples were preserved in an ice pack insulating container to preserve the temperature (4°C) and then transported to the Centre for Genetic Studies laboratory at Maulana Abul Kalam Azad University of Technology (formerly University of West Bengal), Kolkata, India. However, the blood samples were analyzed two (2) days after collectionand they were kept frozen at -20°C due to logistic problems.

3.4.1.2Sample collection for cytogenetic analysis

All cytogenetic analyses wereperformed at the Center for Genetic Studies Laboratory located at the Department of Biotechnology, Maulana Abul Kalam Azad University of Technology, Kolkata, India.

3.4.1.3Sample collection

3.4.1.3.1 Protocol of sample collection

i. Two(2) ml of peripheral blood were collected from DS subjects from two different hospitals in Kano in a sodium heparin vacutainers along with request form for the patient's details including clinical information (Appendix III).

ii. Samples were stored at -20°C and taken one day after collection to the cytogenetic laboratoryCenter for Genetic Studies Laboratory located at the Department of Biotechnology, Maulana Abul Kalam Azad University of Technology, Kolkata, India in an ice box (Vaccine carrier box VC 3.4V., Blowkings CE) and stored for 48 hours.

iii. These blood samples were received and registered in the cytogenetic information section. Each sample was given an identification number (ID) before processing.

3.4.1.4 Peripheral blood lymphocytes culture

Two (2 ml) sterile 15 ml centrifuge tubes were labeled for each sample (duplicate cultures). 0.4ml of heparinized blood was inoculated into 5ml of RPMI-1640 medium substituted with 20% Inactivated Fetal Bovine Serum. 100µl of Phytohemagglutinin (PHA) was added in each culture tube and mixed properly. Culture tubes were incubated at 37° for 72 hours.

3.4.1.5Harvesting the culture

100µl (10 µg/ml) of Colcemid was added into culture tubes and incubated at 37ºC for 50 minutes. Culture tubes were placed in a centrifuge at 500g for 5 minutes; subsequently the supernatant fluid was removed with the aid of 7 ml pasture pipette. The deposit resuspended in 5-6 ml of 0.075 mM prewarmed potassium chloride. Culture tubes were placed in a centrifuge at 500 g for 5 minutes, subsequently the supernatant fluid was removed with the aid of 7 ml pasture pipette (Step 2 was repeated). Using a Pasteur pipette 6ml of a cold fixative (1:3 Acetic acid: Methanol) was slowly added to the pellet while agitating constantly on a vortex mixer. The suspension was then stored overnight at 4ºC before slide preparation (Al-Harasi, 2010).

3.4.1.6Preparation of slides:

Before preparation of slides, they were scrupulously clean; therefore, a suitable cleaning procedure by soaking the slides in 70% ethanol overnight after which they were wash in running water for at least 15 minutes and stored in distilled water at 4ºC.

3.4.1.7Protocol of preparation

The suspension was centrifuged at 1700 rpm for10 minutes and supernatant discarded and the pellet re-suspended with 6 ml cold fresh fixative (1:3 Acetic acid: Methanol).The suspension (Step 1) was centrifuged at 1700 rpm for 10 minutes and supernatant discarded and the pellet re-suspended with 6 ml cold fresh fixative (1:3 Acetic acid: Methanol)was repeatedtwice and finally the pellet was resuspended in 0.5ml of fixative solution and the suspension is ready for slide preparation. 1-2 drops of the suspension were dropped onto a very cold wet slide and allowed to spread using a hot steam from a water bath. Then slides were labeled with the ID number of the patient and date of preparation. Using a phase contrasts microscope slides were checked for a proper metaphase index and spreading (chromosome quality). Finally, all slides were placed in an oven at 65 ºC overnight. (ISCN, 2013).

3.5. GiemsaBanding ("GTG" Banding)

3.5.1. Procedure for staining with GTG banding

For cytogenetic routine samples GTG banding technique (Seabright, 1971) was used at 400 resolution band for diagnosis of all trisomies including trisomy 21.

The following procedure was used for staining:

Prepared slides were removed from oven one hour before banding. Then slides were placed in coupling jar containing 0.2mg/ml trypsin solution starting with 10-20 seconds of incubation. Afterwards, slides were dipped into 1% normal saline to arrest trypsin activity. Slides were placed in Giemsa solution for 5-6 minutes. The slides were rinsed with double distilled water, dried and mounted using DPX mounting medium and cover slips (46x46 mm size). Slides were examined for a proper band quality using Axioskop microscope BX 40 (Karl zeiss) with 100x objective (Oil immersion).

3.5.2. Karyotyping and chromosome analysis

3.5.2.1 Staining procedure

The staining procedure was done in a 37^{o}C water bath. The slides were put in a staining rack (e.g. coupling staining jar) and treated as follows: 2 minutes in 50ml Hanks solution. 1.47 minutes in: 47.5ml Hanks solution + 2.5ml Trypsin-EDTA solution. Wash in: 40ml Hanks solution +10ml fetal bovine serum. Wash in 50ml Hanks solution. 1.5 minutes in: 47ml Buffersolution pH 6.8 + 3ml Giemsa stain solution. Wash several times with 50ml Buffer solution pH 6.8. Air dry the chromosome slides. Check for chromosome spreads in a phase contrast lab microscope.

The prepared slides were examined, karyotyped and analyzed with an Olympus microscope (BX50). A cytogenetic coordinate sheet was used to document patient's details including first name, second name, tribe name, date of birth, lab code number, clinical information, number of cells counted and analyzed and then designation of the karyotype according to the ISCN (1995). After cases were analyzed microscopically, 4 images of metaphase cells were captured and karyotyped using Ikaros meta karyotype system which is composed of an Olympus microscope

BX50 attached with CCD Cohu camera model #4912-5110/0000 and was connected to a computer with a software Smart capture VP (1.4 version specific for karyotyping).

Plate I: Animal cell culture room for karyotyping in MAKAUT, Kolkata, India. Cell culture room is used to multiply experimental model in cellular cell lines and to grow cell in a favorable condition.

3.6. Genotyping of Chromosome 21 with Microsatellite Markers

3.6.1. Samples collection

Samples collection was based on a hospital study in Kano metropolis, Kano State. All cases of Down's syndrome were diagnosed at the Murtala Muhammad Specialist and Hasiya Bayero Paediatric Hospitals, Kano State. Cytogenetic and molecular analyses were performed at Centre for Genetic Studies, Maulana Abul Kalaam Azad University, Kolkata, India. The DNA sample

was selected from proband and the criteria of collection was based on filling a questionnaire structured: Socio-demographic, history of women pregnancies, detailed familial history for two generations, certain events at the conception of the DS child, health and illnesses, X-ray diagnostic, treatments, and occupational history in addition to obtaining DNA blood samples from the Down's child. The total number of samples collected was 16 cases**.**

3.6.1.1. Protocol of sample collection:

Two ml of peripheral blood lymphocytes (whole blood) samples were collected in sterile sodium-EDTA vacutainer tubes for molecular detection of DS. The DNA extraction was performed by using BioRobort DM48 of Qiagene Company.

3.6.2. DNA extraction

The procedure used for the extraction of genomic DNA was isolated from uncoagulated blood samples of trisomic proband by using salting out procedure of Miller *et al*. (1988). DNA bound to the magnetic particles is then washed with two different buffers followed by a rapid rinse with distilled water which considerably improves the purity of the DNA.

3.6.2.1. DNA extraction protocol

A total of sixteen blood samples were collected and kept in 4°C and subjected to DNA extraction using modified salting-out method. In this method 500 µl blood was used and the following procedure was adopted: 1.5 ml of R.B.Cs lyses buffer (155 mmol ammonium chloride, 10 mmol potassium hydrogen carbonate, 1mmol EDTA, adjust PH to 7.6) was added to the blood samples. Tubes were incubated on ice 15 minutes inverting occasionally.

The blood was centrifuged at 4500 rpm for 15 minutes and the supernatant was discarded. To the remaining white pellet resuspended in the residual supernatant, 1.5 ml of the proteinase K buffer (20 mmol Tris-HCl, 4mmol Na2EDTA, 100 mmol NaCl,adjust PH to 7.8) was added, also 100 µl of 10% Sodium dodecyl sulphate (SDS) was added. The solution mixed until the pellet was re-suspended and well dissolved. Then 20 µl of fresh, refrigerated Proteinase K solution (20mg/ml) was added. The tubes were placed in a water bath at 55C° for 90 minutes. The tubes were placed on ice to cool for 2-3 minutes, 1 ml of 5.3 M Nacl was added and vortexed for 15 second. They were then centrifuged at 4500 rpm for 20 minutes. The supernatant was transferred to a new set of tubes containing equal volume of cold isopropanol. The tubes were inverted 5-6 times gently to precipitated DNA, then centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded and ethanol (70%) was added, again tubes centrifuged. The supernatant discarded and 200-300 µL of distil water was added to re-suspended DNA.

The Optical densities of the DNA samples were obtained by adding 50 µL of the DNA prepared solution into tubes containing 950 µL of diluent. Each of the new solutions were added to quartz cuvettes that were placed in a spectrophotometer and the optical densities were tabulated using

distilled water as the blank control. The 260 nm / 280 nm ratio was obtained to give an analysis of the purity of the sample and the concentration of the extracted DNA was found.

3.6.3. STR-PCR master mix and conditions

PCR amplification were performed in a total reaction volume of 100 µl. The primers were diluted to 10µM. To perform PCR reaction, a master mix was prepared containing water, buffer (10X PCR buffer with $(NH4)_2SO_4$), dNTPs (Fermentas), reverse and forward primers and Taq DNA polymerase (Fermentas) in a single tube, which was thenaliquoted into individual tubes containing template DNA. Optimized concentration of $MgCl_2$ (Fermentas) was then added.For the primer, after initial denaturation at 94^0C for 5 min., 29 cycles of PCR amplification were done at 94^0C for 40s; 59^0C for 30s; 72^0C for 40s and final extension for 5 min at 72^0C. PCR (polymerase chain reaction) reactions were carried out in a Perkin-Elmer Gene Amp.2400 Thermal Cycler. The amplified DNA were analyzed in 8% polyacrylamide gel and analyzed in UV transilluminator after staining with ethidium bromide.PCR products were analyzed by following the method described earlier by Ghosh and Dey (2003).Primer hybridization specificity was checked against the human genome using BLASTn.

3.6.4. DNA PAGE gel electrophoresis

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'-methylenebisacrylamide. The reaction is a free radical polymerization, usually carried out with ammonium persulfate as the initiator and N, N, N', N'-tetramethylethylenediamine (TEMED) as the catalyst. Although the gels are generally

more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have agreater resolving power, can accommodate larger quantities of DNA without significant loss in resolution and the DNA recovered from polyacrylamide gels is extremely pure (Guilliatt, 2002). Moreover, the pore size of the polyacrylamide gels can be altered in an easy and controllable fashion by changing the concentrations of the two monomers (Budowle and Allen, 1991).

3.6.4.1. DNA PAGE gel electrophoresis procedure

Polyacrylamide gels are poured and run in 0.5X or 1x TBE at low voltage (1-8 V/cm) to prevent denaturation of small fragments of DNA by heating. The gel was run slowly in 1x TAE, which does not provide as much buffering capacity as TBE. Glass plates and spacers were cleaned thoroughly. The plates were held by the edges and gloves were worn, so that oil from the hands do not become deposited on the working surfaces of the plates. The plates were rinsed with deionized water and ethanol and were set aside to dry. The glass plates assembled with spacers in gel caster. Gel solution was prepared with 8% polyacrylamide percentage, 0.4 g bisacrylamide, 48 g urea, 10× TBE buffer, 40 ml deionized water, 10% of 200(µl)APS, 10 µl Tetramethylethylenediamine (TEMED). The TEMED was added to complete the gel before the acrylamide polymerizes.Immediately appropriate comb was inserted into the gel, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth were slightly higher than the top of the glass. The comb was clamped in place with bulldog paper clips. The remaining acrylamide gel solution was used to fill the gel mold completely. Make sure that no acrylamide solution is leaking from the gel mold.The acrylamide was allowed to be polymerized for 30-60 minutes at room temperature.After polymerization was completed, the comb was

surrounded and the top of the gel with paper towels that have been soaked in 1x TBE. The entire gel was sealed in Saran Wrap or plastic bag and stored at 4°C. The gels were removed from the gel caster, spilled and cleaned and inserted in the Hofer box. A running buffer was added, and the combs were carefully pulled from the polymerized gel. A Pasteur pipette was used to flush out the wells once more with 1x TBE. The DNA samples were mixed with the appropriate amount of gelloading buffer. The mixture was loaded into the wells using a micropipette equipped with a drawn-out plastic tip.The electrodes were connected to a power pack, turned on the power, and begin the electrophoresis run for 30 minutesat 5 V/cm (constant voltage). The gel was run until the marker dyes migrated to the desired distance. Then theelectric power was turn off, the leads were disconnected, and the electrophoresis buffer was discarded from the reservoirs.The glass plates were detached and laid on the bench. A spacer was used to lift a corner of the upper glass plate. The gel was checked and remained attached to the lower (white) plate. The upper plate was pulled smoothly away, and spacers were removed. The gels were stained with ethidium bromideand exposed to PhosphoImager screen.

3.6.5. Primer used

Primers used in this research were specifically for microsatellite markers on chromosome 21. Primers were oligonucleotides complementary to the 5' and 3' sequences flanking microsatellites. The sequence and PCR conditions for the primer were as follows: forward primer,5´GTGAGTCAATTCCCCAAG3´ (only the 5' end of forward primers were fluorescent dye-labeled) and reverse primer, 5´ GTTGTATTAGTCAATGTTCTCC3´.

3.6.6. Primer design

Primers used were designed from Human Genome Database (HDG), National Centre for Biotechnology Information (NCBI). Primer pairs for the detection of STR markers on chromosome 21 were obtained from Xcelris Genomics.

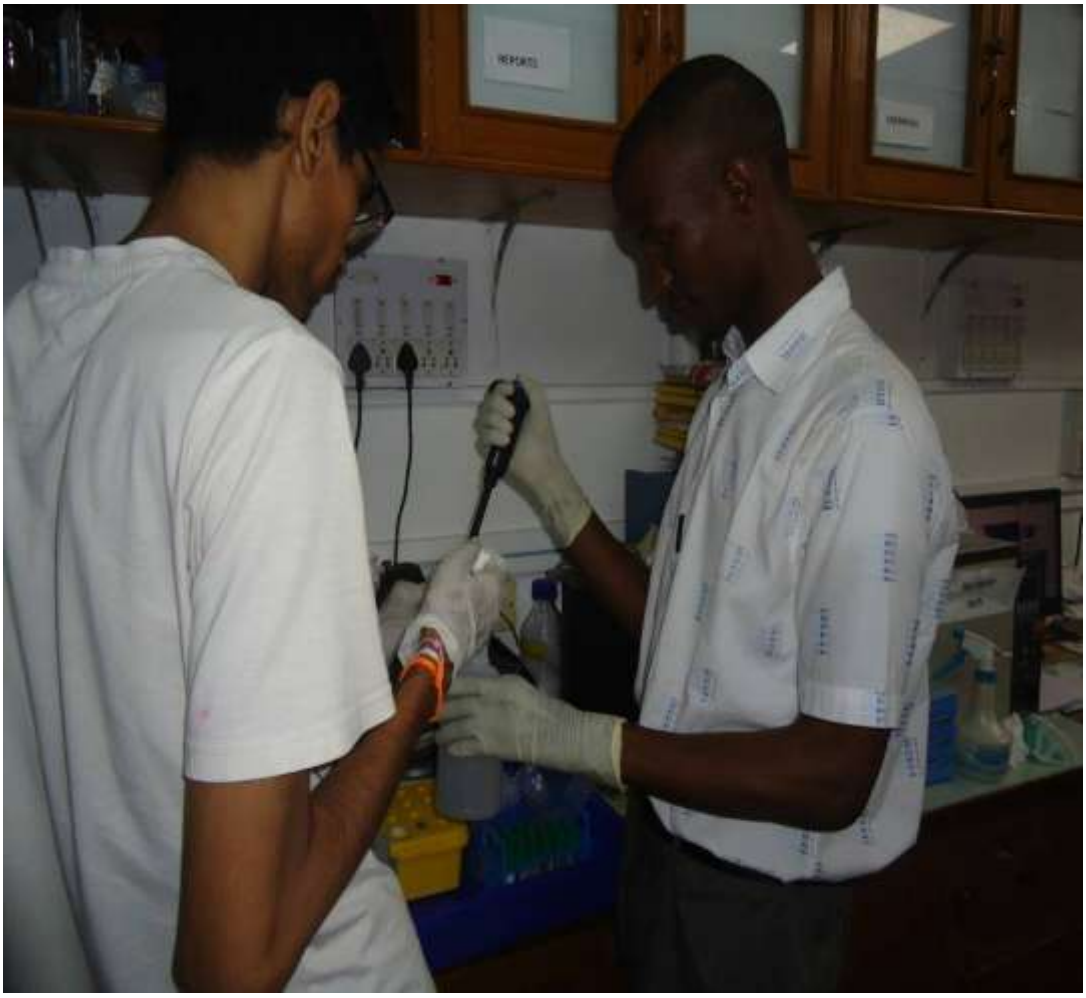


Plate II: Molecular study roomfor DNA extraction at Centre for Genetic Studies Kolkata, India

3.7 Data Analysis

The data were analyzed and expressed as Mean ± SD, frequency and percentages. Pearson's Chi square test was used to determine the association of clinical features (facial features) with status of the participants (DS and control). Sexual dimorphism in frequency of DS and association of maternal age with DS was also tested using Pearson's Chi square test. Box and whisker plot were used to determine the association of DS type and parental age. Receiver operating

characteristic curve was used to discriminate between the DS patient and control using maternal and paternal ages. The analyses were carried out using SPSS version 20 and $P< 0.05$ was set as level of significance.

CHAPTER FOUR

4.0 RESULTS

4.1 Results of Cytogenetic Analyses

The clinical diagnosis of Down syndrome was confirmed by chromosome analyses and karyotyping after Giemsa (GTG)-banding for all Down syndrome patients. Subsequently, the

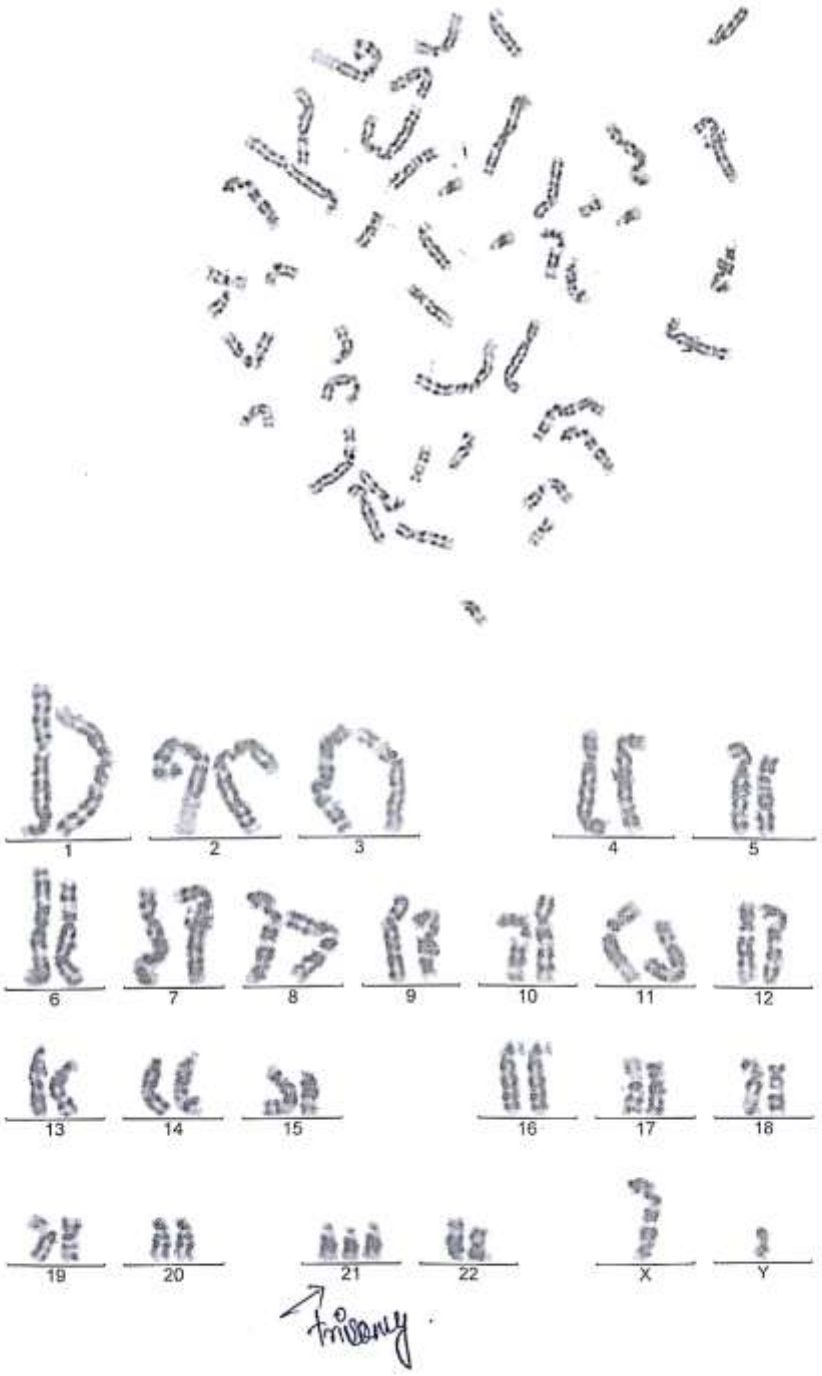
frequency of different types of cytogenetic aberrations found in Nigerian DS children was determined.

4.1.1 Results of the chromosomes analyses

The cytogenetic results of the analyses of 16 cases of Down syndrome are presented in plates III-IV. Non-disjunction of trisomy 21 and hence free trisomy 21 was the only most common type of abnormality detected in 100% (N=16) of the cases. There were no cases of translocation and mosaicism trisomies in all the cases confirmed cytogenetically.

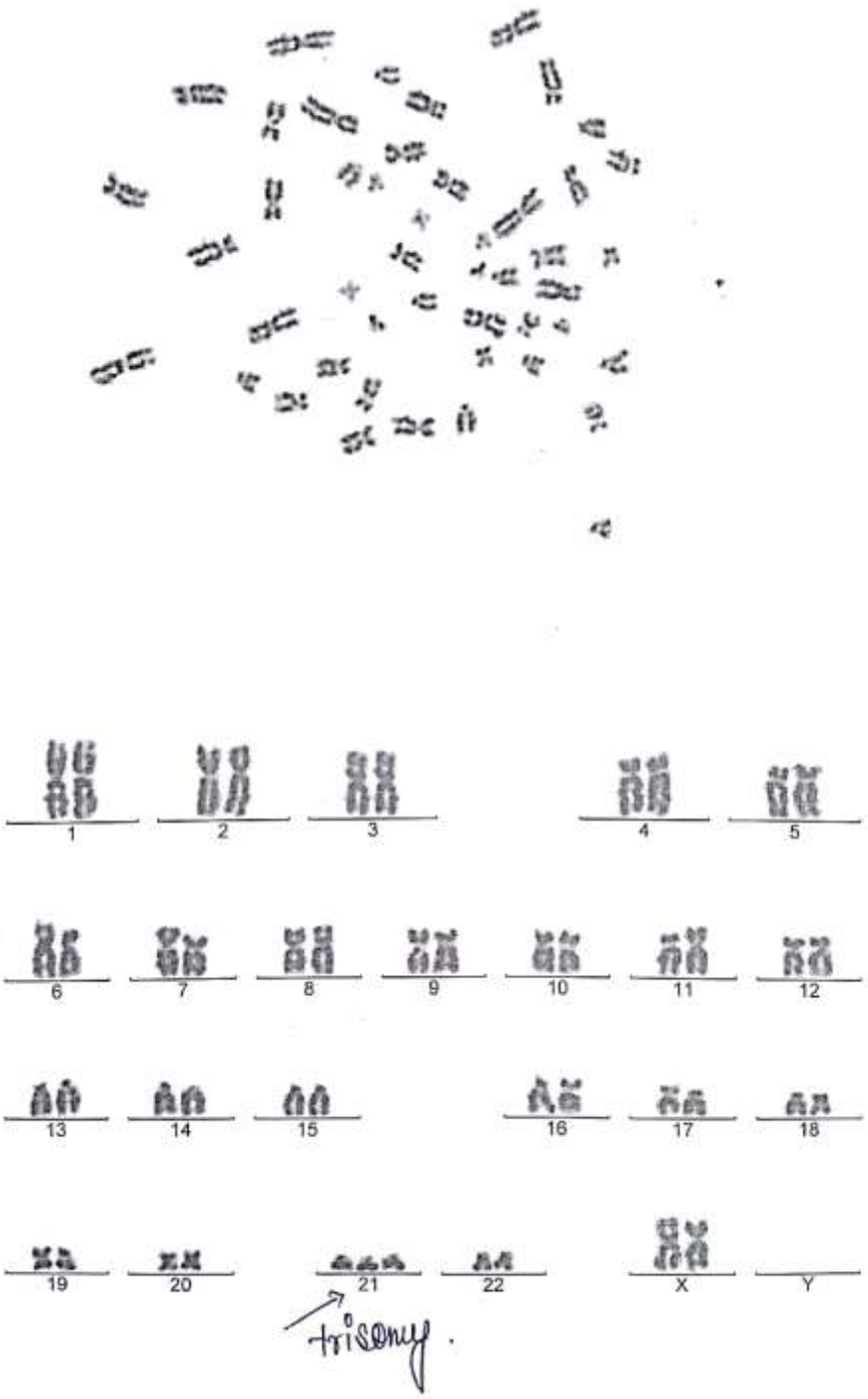
Among karyotyped cases listed there were 9 males and 7 females with a percentage of 56.25% and 43.75% respectively (Table 4.1).

There was a significant excess of males observed in all the group with free trisomy 21 with a sex ratio of 1.3:1,while there were nocases of sex ratio with translocations and mosaicism respectively (Table 4.2).



Plate

II1: A karyotype showing a male with trisomy 21 after capturing with Ikaros meta system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21



PlateIV: A karyotype showing a female with trisomy 21 after capturing with Ikaros meta system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21.

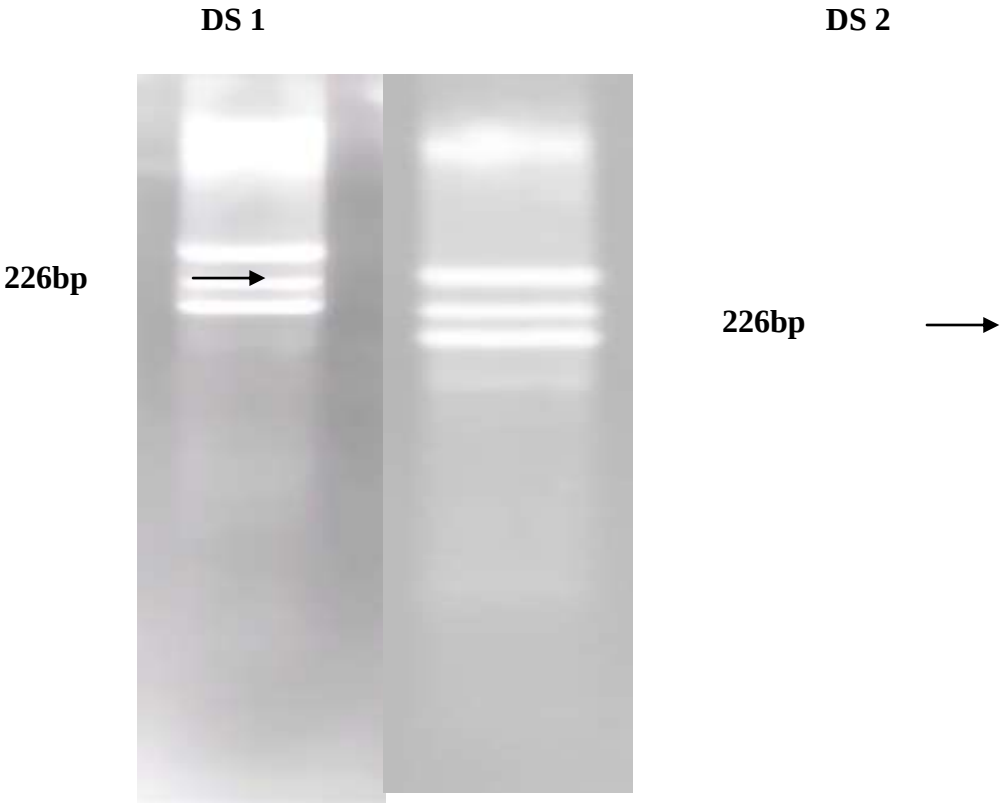
Table 4.1: Results of karyotype analysis for 16 Down syndrome cases

Karyotype result	Number of cases	%
Free trisomy	16	
47, XX, +21	7	43.75
47, XY, +21	9	56.25
Translocation	-	-
Mosaic	-	-
Total	16	100

4.1.2Molecular Studies

The molecular analysis involves PCR amplification of small tandem repeat (STR) markers located on human chromosome 21 and analysis by fluorescence based method by ethidium bromide to identify the presence of an additional allele on the third copy of the chromosome so thus confirming the cytogenetic analyses.

The gel pictures shows STR pattern of D21S11 marker in DS patients (Plate V). Out of 16 families analyzed we detected trisomy in 100% cases. DS 1 and DS 2 connotes male and females DS patients. All of the probands had chromosome 21, but the parental origin was not specified in all the DS families. Thus, the analysis using the microsatellite markers D21S11 was informative on the detection of trisomy 21 only on DS children.



PlateV.Gel pictures showing STR pattern of marker D21S11 on Chromosome 21. DS1is showingmale and DS2female DS patients.(NB: Analysis of chromosome and karyotype revealed a diploid count of 2n=47, +21 in probands of all DS families).

4.1.3 Ages at diagnosis and birth order of DS subjects

Sixty (60%) percent of the children with DS were diagnosed at less than one year of age (< 1 year), 32% were diagnosed between 1-2 years, while 8% were diagnosed after 2 years (Figure 4.1). Thus, almost 90% of DS children born in Kanowere not diagnosed cytogenetically within 6 months after birth.

Figure 4.2 shows the comparison of birth order between normal subjects and DS patients of study population. A significant association (X^2= 0.965, p=0.915) was observed between the DS and birth order. The higher the birth order the more chances of given birth to a child with DS. Birth order was found to be higher in the control between second (2%) and middle borns (11%) when compare with DS cases (1%) and (9%) respectively. There was a higher number of DS children (case) in the last birth order (21), when compared to the control (19%).However, a sudden rise in the frequency of DS children was also noted from 1stbirth order.

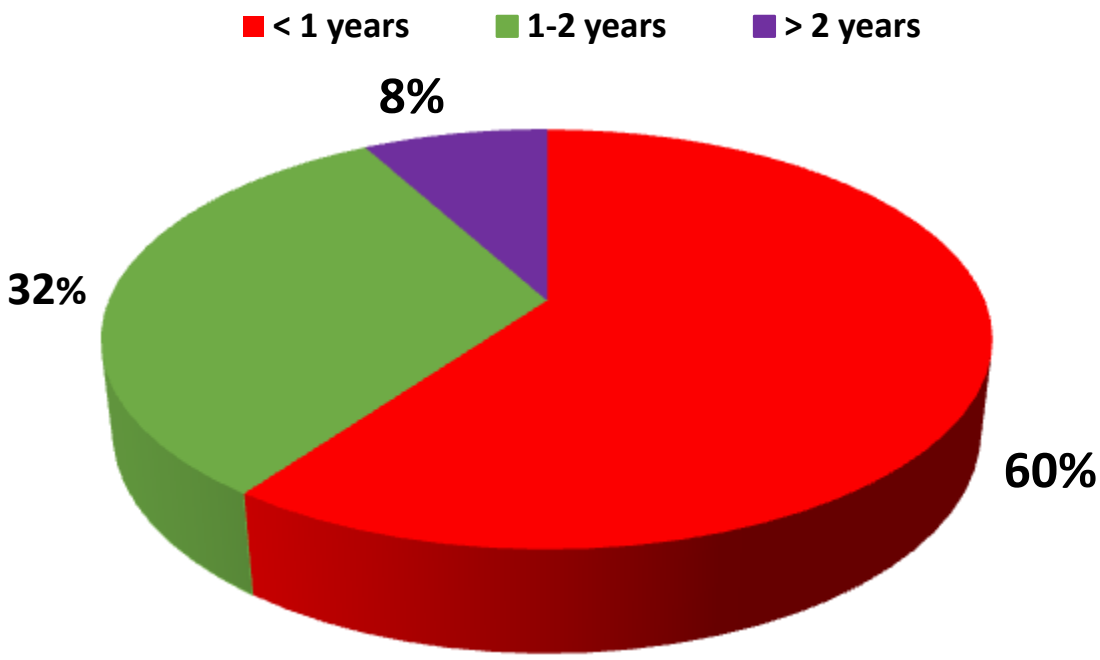


Figure 4.1: Age at referral of postnatal Down syndrome cases

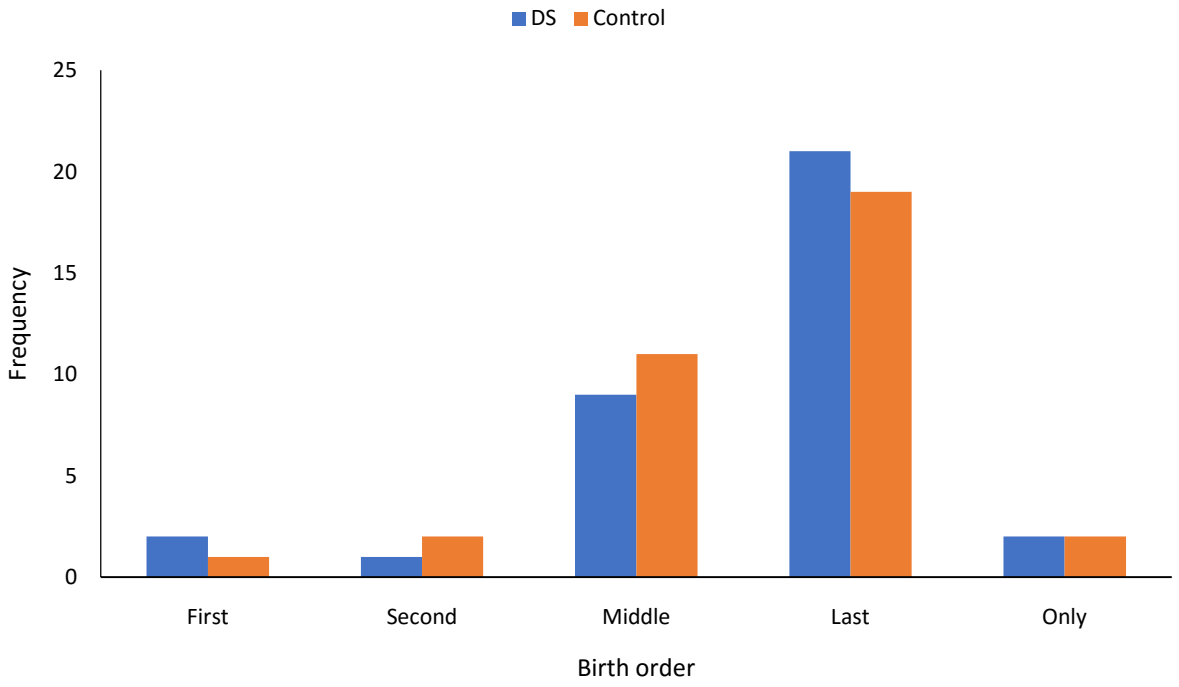


Figure 4.2: Comparison of birth order between control subjects and DS patients of study population(X^2=0.965, df=4, *p*=0.915)

4.2 Case Control Study and Results from the Questionnaire

4.2.1. Results of the case control study

The relationship of some clinical features of DS and controlin the study population (Table 4.3 and 4.4).The cases were matched with the control for the birth of a DS child respectively within the same year of birth and from same health region. It was observed that there was a significant upward slanting fissure in DS compare with control. Similarly, lower set ears, microglossia, and short neck were found to be significantly associated with DS patients compared to the control. There was a significant flat occiput and microcephaly in DS compare with control (Table 4.3).

Similarly, simian crease, hypotonia, and mental retardation were found to be significantly associated with DS patients compared to the control. It was observed that congenital heart disease particularly atrioventricular septal defect (AVSD) was associated with DS compared to the control. However, hypothyroidism, associated medical conditions, and pes planus shows statistically significant differences between DS patients when compared to the control (Table 4.4)

Table 4.2. Relationship of some clinical features between Down syndrome subjects and control

Clinical features	Status	DS (EF)	Control (EF)	$\chi 2$	p-value
Upward slanting fissure	Yes	35 (17.5)	0 (17.5)	70.00	< 0.0001
	No	0 (17.5)	35 (17.5)		
Low set ears	Yes	35 (17.5)	0 (17.5)	70.51	< 0.0001
	No	0 (17.5)	35 (17.5)		
Microglossia	Yes	33 (17.5)	0 (16.5)	62.43	< 0.0001
	No	2 (1.4)	35 (18.5)		
Short neck	Yes	33 (16.5)	0 (16.5)	62.43	< 0.0001
	No	8 (7.5)	25 (25.5)		
Flat occiput	Yes	35 (17.5)	0 (17.5)	70.00	< 0.0001
	No	0 (18.0)	35 (18.0)		
Pes planus	Yes	34 (17.0)	0 (17.5)	6.11	< 0.0001
	No	1 (18.0)	35 (18.0)		

Table 4.3. Relationshipof some clinical features between Down syndrome subjects and control

Clinical features	Status	DS (EF)	Control (EF)	$\chi 2$	p-value
Simian crease	Present	35 (17.5)	0 (17.5)	70.00	< 0.0001
	Absent	0 (17.5)	35 (17.5)		
Hypotonia	Present	29 (14.5)	0 (14.5)	49.51	< 0.0001
	Absent	6 (20.5)	35 (20.5)		
Mental retardation	Present	35 (17.5)	0 (17.5)	70.00	< 0.0001
	Absent	0 (17.5)	35 (17.5)		
Congenital disease	Present	8 (4.0)	0 (4.0)	9.03	< 0.0001
	Absent	27 (31.0)	35 (31.0)		
Hypothyroidism	Present	2 (1.5)	0 (1.0)	2.06	< 0.0001
	Absent	33 (34.0)	35 (34.0)		
Pes planus	Present	1 (1.5)	0 (0.5)	1.014	< 0.0001
	Absent	34 (34.5)	35 (34.5)		

4.2.2 Congenital heart defects of DS cases

The prevalence of CHD among DS subjects was grouped into DS children with CHD and those without CHD. The results show 20% of DS children have CHD while 80% ofDS are children without CHD (Figure 4.3).

Of the thirty-five (35) children with clinical cases of DS, 8 (4%)children had CHD.Of the 8 cases 5 (57%) hadatrioventricular septal defect (AVSD) and 3cases(43%) had atrial septal defect (ASD) (Figure 4.4).

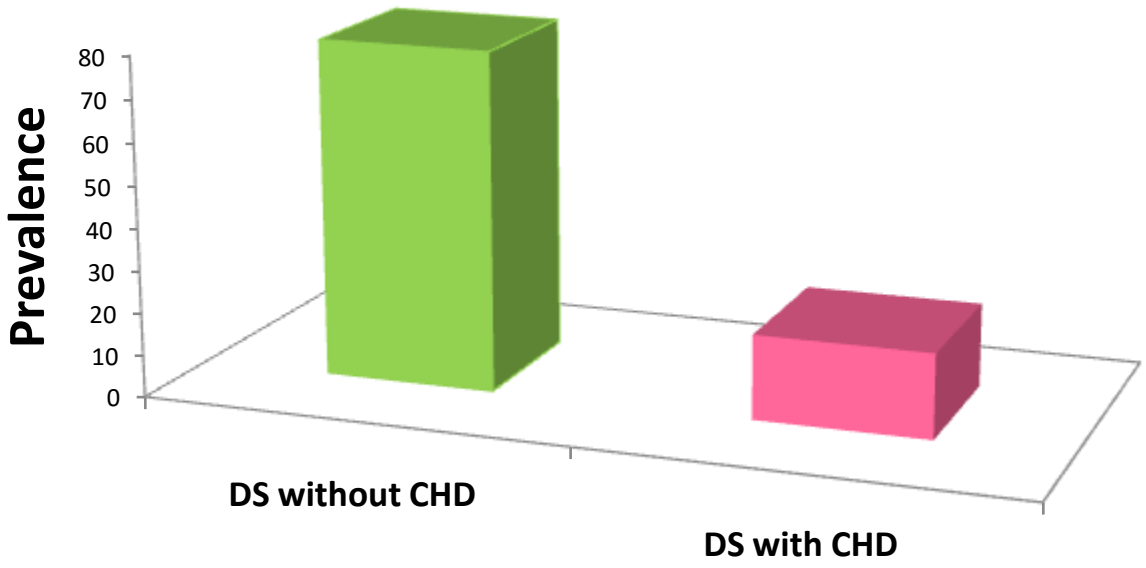


Figure 4.3: Prevalence of congenital heart defectamong DS subjects

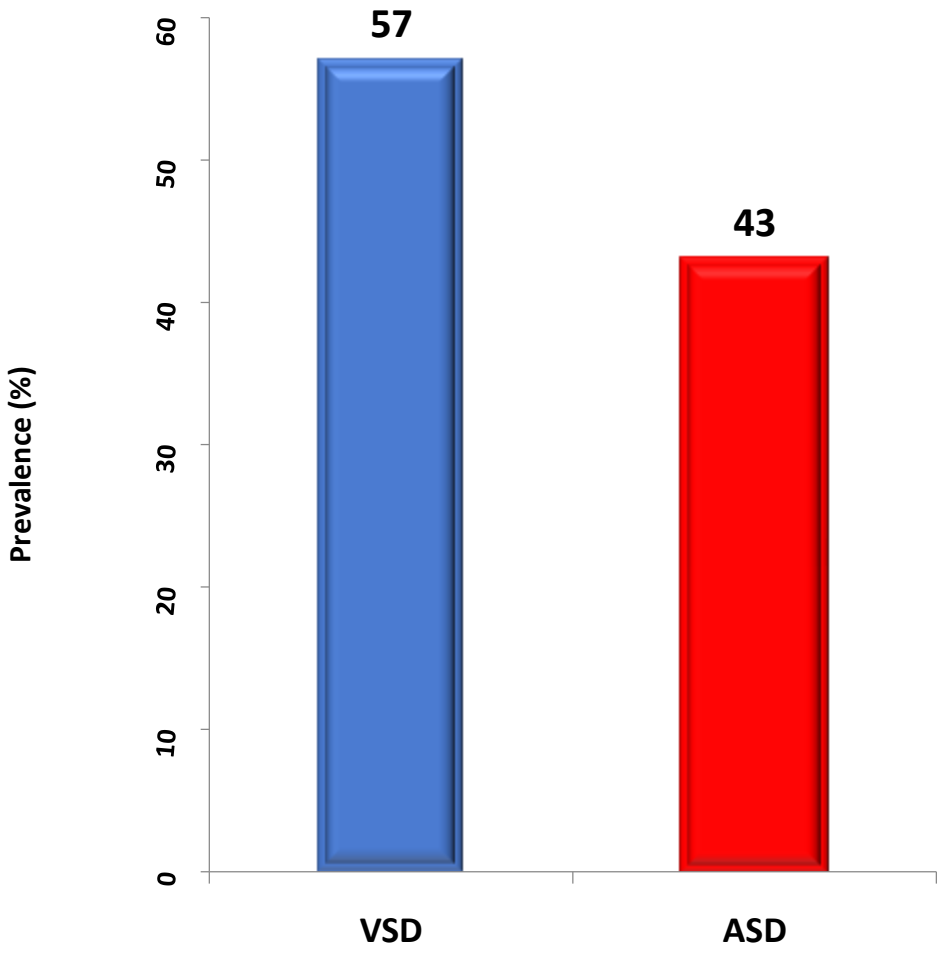


Figure 4.4: Types of congenital heart defects among Down syndrome subjects
(AVSD= Atrioventricular Septal Defect; ASD= Atrial Septal Defect)

4.2.3 Maternal ages of mothers of DS and control cases

The mean maternal ages of control and DS cases (Figure 4.5). There was an increase in mean maternal age of DS mothers of 35.66± 8.53 years. This mean age was higher than the maternal age of mothers of non-trisomic children, whose age was around 31.28 ± 5.96 years.

The frequencies of birth of DS children with maternal age (Figure 4.6). The ages were divided into five (5) groups.Group I (< 25 years), Group II (25-30 years), Group III (31-35 years), Group IV (36-40 years) and Group V (> 40 years). Group II (25-30 years) shows a sudden increase of 80% in the control mothers as compared with DS mothers. Group III shows a 60% decrease frequency in DS mother as compared to the control mothers with 90%. In Groups IV and V, there were 50% and 80% frequency of increased birth cases of DS is higher with advanced maternal age. Older age (maternal) is associated with increased number of DS cases.

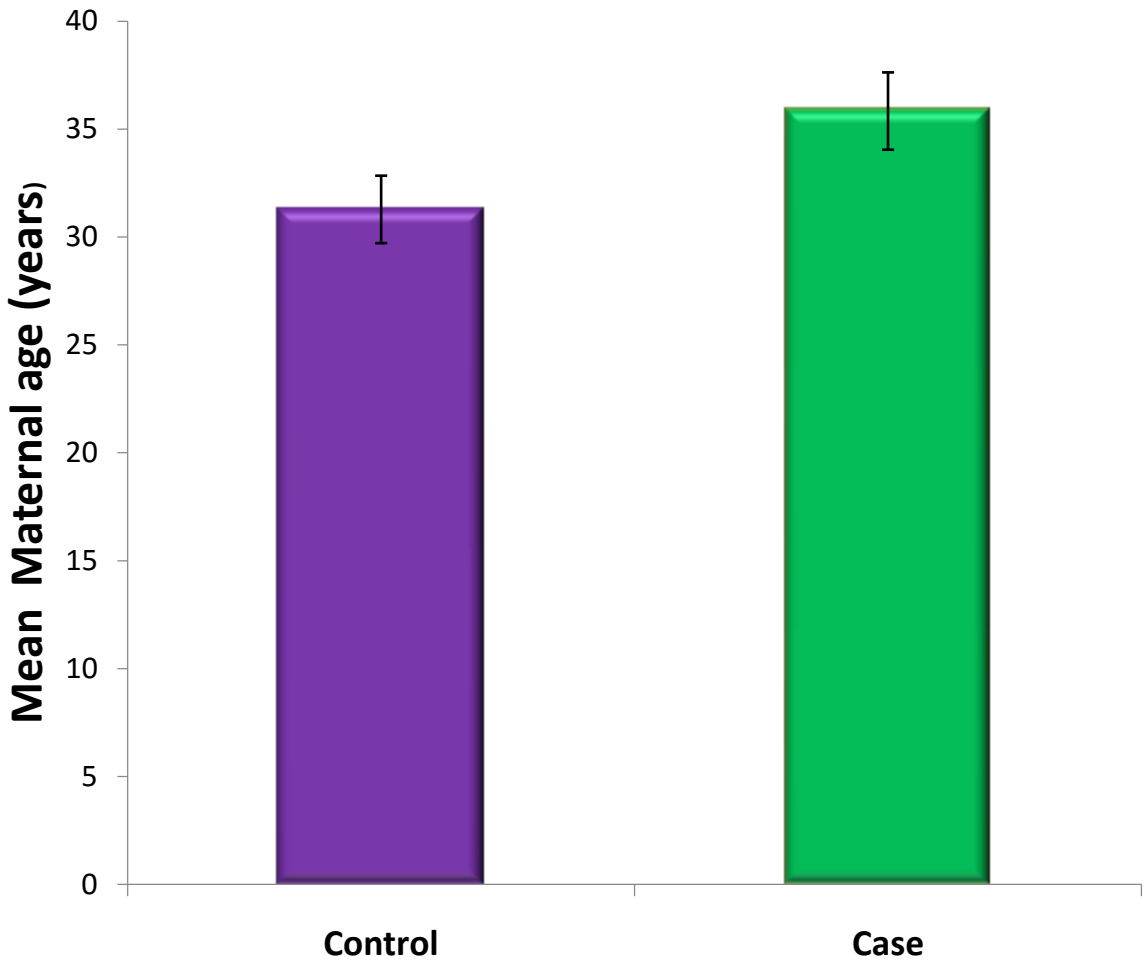


Figure 4.5: **Mean maternal ages of control and DS cases(P=0.077)**

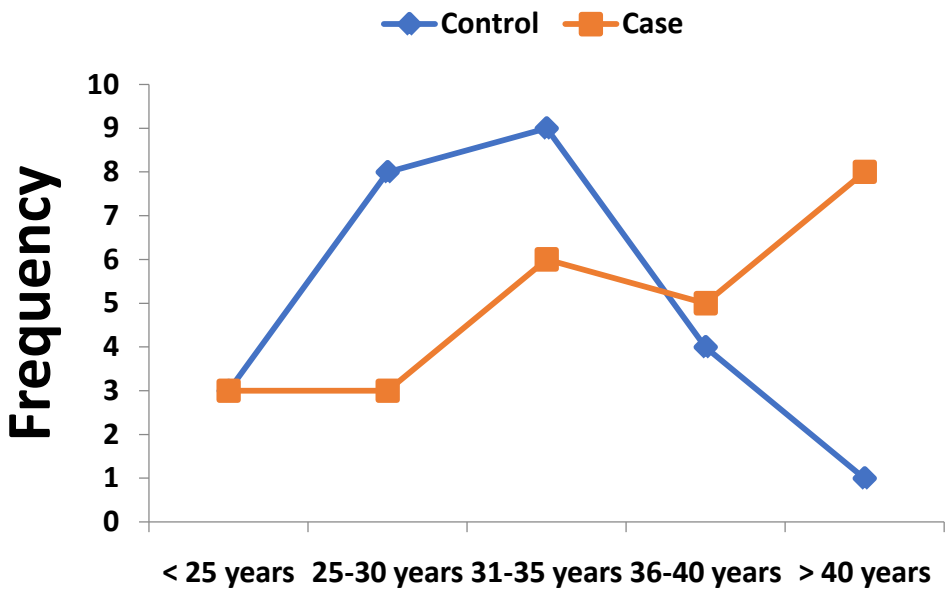


Figure 4.6: **Frequencyof maternal ages between DS mothers and control**

4.2.4Parental age at time of DS birth

The age of the mothers and fathers of the trisomy 21and controlsubjects (Figure 4.7). There was a significant difference of maternal and paternal ages between DS patients and non trisomic subjects or controls. In both maternal and paternal ages of DS patients, the mean age is higher compared to that of control. It was observed that the maternal age was significant (Area under curve (AUC)= 0.67, p=0.025) as a risk factor for giving birth to DS patients. A cut-off value of 42 years and above indicates the risk of giving birth to child with DS (Sensitivity = 0.31, Specificity= 1.00).

However, the paternal age does not play any significant role (AUC= 0.62, p=0.132) as a risk factor for giving birth to DS patient, but 47 ± SD years was found to have the best sensitivity and specificity in discriminating (Sensitivity = 0.51, Specificity= 0.84) between the two groups.

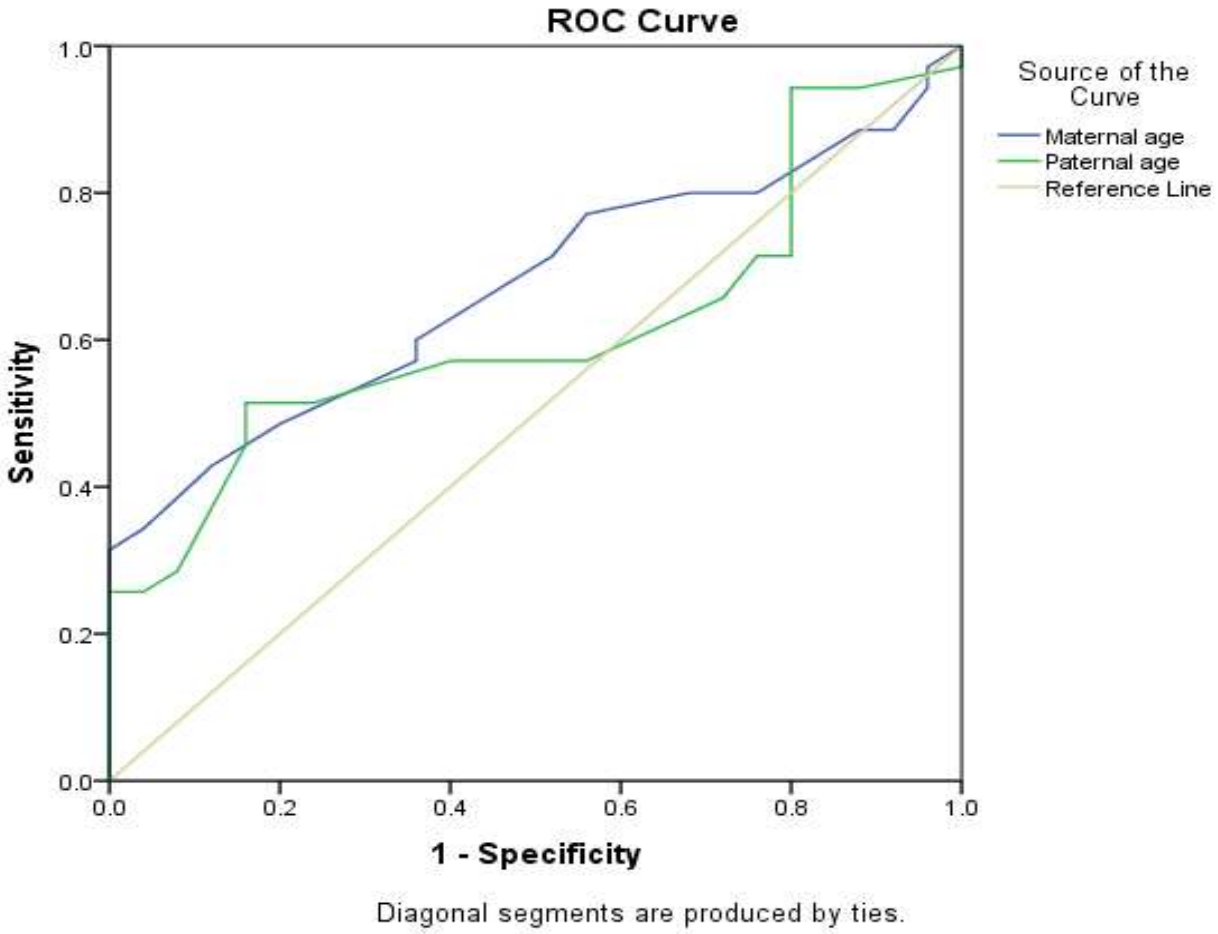


Figure 4.7:Receiver operating characteristics curve for discriminating the role of parental age as a risk of giving birth to normal child and DS patients.

4.3. Socio-demographic Results

4.3.1 The Frequency distribution of sex and birth order in relation to CHD of DS subjects

The Frequency distribution of sex and birth order on occurrence of CHD of DS subjects (Table 4.5). Thirty-five cases of were recruited into the study. Among them, eight (8) had CHD. It was found that 3% of male DS cases had CHD, whereas only 5% of female cases had CHD. Similarly, for birth order, 2% of DS of the middle born had CHD. In the last birth order, 5% cases had CHD, whereas only born had 1% case (0.5) of CHD. No associations between sex, birth order and the type of CHD (p= 0.392; $\chi 2$=0.73; p=0.844; $\chi 2$=2.07, respectively) were found.

Table 4.4: Frequency distribution of sex and birth order on occurrence of CHD of DS subjects

		Congenital heart diseases (CHD)				
		CHDOF(EF)	No CHDOF(EF)	n=35	χ2/FEV	p-value
Sex	Male	4 (3)	18 (17)	22	0.73	0.392
	Female	4 (5)	9 (10)	13		
Birth order	First	0 (0.5)	2 (1.5)	2	2.07	0.844
	Second	0 (0.2)	1 (0.8)	1		
	Middle	2 (2)	7 (7)	9		
	Last	5 (5)	16 (16)	21		
	Only	1 (0.5)	1 (1.5)	2		

OF = Observed frequency; EF= Expected frequencyFEV= Fishers Exact Value (for association of birth order and CHD)

4.3.2 Association and comparison of parental age and DS type in relation to CHD

The association between parental age and CHD in children with DS subjects (Table 4.6). In the maternal age group, mothers > 25 years had 2 (0.9%) of children. In the age group of 25-30 years, 0 (0.9%) of DS children had CHD. When the maternal age was 31-35 years 1 (2.3%) of DS children had CHD and 36-40 years mothers of DS children had CHD 1(1.1%). When the maternal age was above 40 years the chances of CHD increased to 4 (2.7%) in DS children with CHD (The p value of 0.352 is not statistically significant).

In the paternal age group of 25-30 years, the risk of CHD in Down syndrome offspring is 0.5%. But in the age group of 31-35 years the risk rises to 1.8% and when the age is 36-40 years the risk is 1.1% and when the age is 40 years and abovethere is 4.6% chances of CHD in DS children.(The p value of 0.237 is not statistically significant).

Table 4.5: Association of parental age with congenital heart defect of DS subjects

		Congenital heart diseases (CHD)				
Parental age		CHD (EF)	No CHD (EF)	n=35	FEV	p-value
Maternal	<25	2 (0.9)	2 (3)	4	4.11	0.352
	25-30	0 (0.9)	4 (3.1)	4		
	31-35	1 (2.3)	9 (7.7)	10		
	36-40	1 (1.1)	4 (3.9)	5		
	>40	4 (2.7)	8 (9.3)	12		
Paternal	25-30	1 (0.5)	1 (1.5)	2	4.08	0.237
	31-35	0 (1.8)	8 (6.2)	8		
	36-40	1 (1.1)	4 (3.9)	5		
	>40	6 (4.6)	14 (15.4)	20		

EF= Expected frequency; FEV= Fishers Exact Value (for association of birth order and CHD)

4.3.3 Frequency distribution of CHD type based on sex

Percentage distribution of CHD type based on sex (Figure 4.10). It was found that femaleswith DS had more cases of AVSD (4%) than the male DS subjects (2%) and ASD was found to be more in male DS subjects (2%) than the female DS subjects (1%).

Figure 4.8: Frequency distribution of CHD type based on sex

4.3.4 Association of some clinical features of DS subjects with CHD

Association of clinical features with CHD (Table 4.7). From the result, macroglossia was found to be present in 7 (7.5%) and absent in 1 (0.5%) of DS with CHD. Similarly, microcephaly was present 7 (7.8%) and absent 1 (0.2) Also, hypotonia was present 7 (6.6%) and absent 1 (1.4%). However, hypothyroidism was present in 0 (0.5%) and absent 8 (7.5%) in DS with CHD. Pes planus was present in 0 (0.2%), absent in 8 (7.7%) in DS with CHD. Also, short neck was present in 7 (7.5%) and absent in 1 (0.5%) in DS children with CHD. There is no association between clinical features of DS and CHD.

Table 4.6: Association of some clinical features of DS with CHD

		Congenital heart diseases				
Clinical features	Status	CHD	No CHD	n=35	X^2	p-value
Microglossia	Yes	7 (7.5)	26 (25.5)	33	0.89	0.346
	No	1 (0.5)	1 (1.5)	2		
Microcephaly	Yes	7 (7.8)	27 (26.2)	34	3.47	0.062
	No	1 (0.2)	0 (0.7)	1		
Hypotonia	Yes	7 (6.6)	22 (22.4)	29	0.16	0.692
	No	1 (1.4)	5 (4.6)	6		
Hypothyroidism	Yes	0 (0.5)	2 (1.5)	2	0.63	0.428
	No	8 (7.5)	25 (25.5)	33		
Pes planus	Yes	0 (0.2)	1 (0.7)	1	0.31	0.581
	No	8 (7.7)	26 (26.2)	34		
Short neck	Yes	7 (7.5)	26 (25.5)	33	0.89	0.346
	No	1 (0.5)	1 (1.5)	2		

4.3.5. Frequency of free trisomy 21 type in association with paternal age

Comparison of DS type and parental age (Figure 4.9). The results were statistically significant in both maternal and paternal ages with free trisomy 21of children with DS (p=0.0001). The median maternal and paternal ages in all DS children was (35.6± 8.53; 46.34± 11.7 respectively).

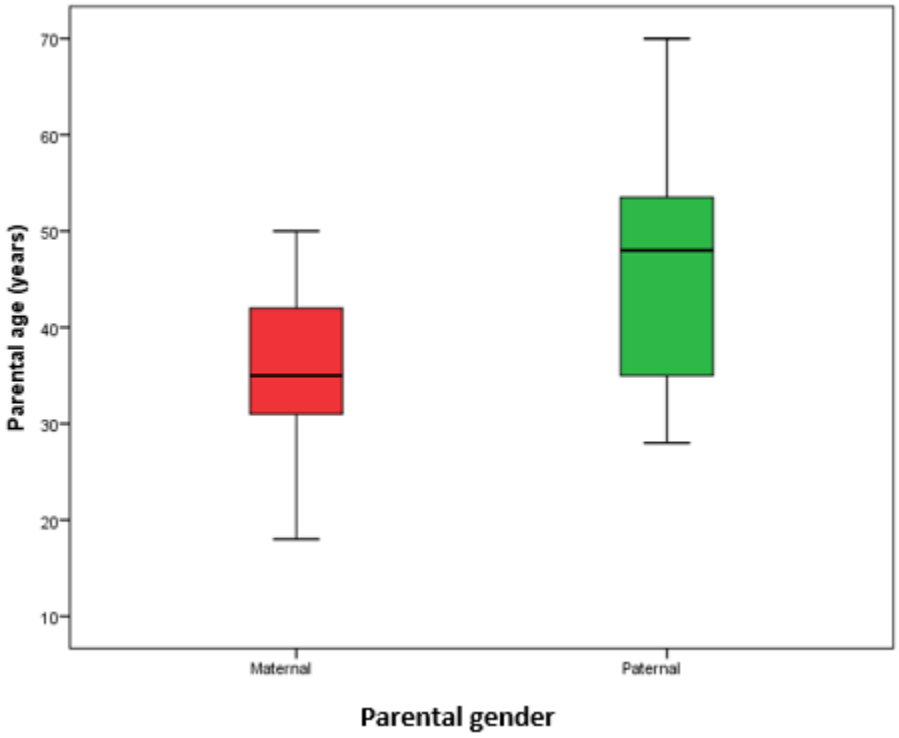


Figure 4.9. Comparison of Down syndrome type and parental age usingMann Whitney test. Box-and-whisker plots present the medians of parental ages in free trisomy 21. Each bar shows upper and lower quartile, while the square and its central bar indicate interquartile range and median. P-probability. (Maternal age= 35.6± 8.53 (Minimum=18.00; Maximum= 50, Paternal age= 46.34± 11.7, (Minimum 28, Maximum= 70), p<0.0001).
NB: Shapiro test of normality indicates normally distributed data for both maternal and paternal ages (statistical value= Maternal 0.963 and 0.289; Paternal = 0.942 and 0.067).

CHAPTER FIVE

5.0 DISCUSSION

Trisomy 21 is a common birth defect and can be diagnosed easily based on clinical features. However, karyotyping is necessary for the confirmation of free trisomy 21, mosaicism, and translocation in DS children to determine the recurrent risk and to provide genetic counseling(Devlin and Morrison, 2004).However, molecular analysis is necessary for the assessment of trisomy 21 and to confirm the presence of additional three alleles of karyotypic pattern of trisomy 21 of children with DS. The data reported in this study represent the first work of DS in Kano State, Nigeria. All cases were diagnosed postnatallyusing karyotypeanalysis and DNA diagnosis of DS using simple PCR and also STR marker.

The frequencies of the different karyotype patterns observed in these subjects are shown in Plates I-II. In our result the percentage of free trisomy 21 was found to be 100%, translocation trisomy 0% and mosaic trisomy 0%. Free trisomy 21 was the only karyotypic pattern that was observed in our study population. This resultagrees with the work of Kava *et al*. (2004) in India, who found free trisomy in 95%, translocation in 3.2% while 1.8% were mosaics.Our results were also similar in terms of free trisomy to the work of Ahmed *et al*. (2005) in Pakistan, who observed in a sample of 295 patients, frequencies of 95.6%, 3.7% and 0.7% respectively for free trisomy, translocation and mosaicism. These frequencies do not significantly differ from those observed by Mutton *et al.* (1996) in England and Wales with 95% for free trisomy, 4% for translocations and 1% for mosaicism in a total of 5737 patients.

In this study, the overall sex ratio was 1.3:1for M: F. The male preponderanceis a universal finding and was reported in many studies of the world. Our results are lower than those found by Kolgeci *et al*. (2013) in Kosovo (1.72:1), and near to those of Amayreh *et al*. (2012) in Jordan (1.61:1).

The higher male to female sex ratio may be the inherent tendency of Y belonging to the G group chromosome, which is closer to its other members, 21 and 22, especially the smallest acrocentric the 21 (Belmokhtar *et al*., 2016). The reasons for the excess of male DS associated with the paternal errors are not yet clearly known (Petersen*et al*., 1993).The excess of males among Down syndrome children has been reported from almost all studies from various countries like England, Wales, Scotland and Italy (Petersen *et al*., 1993; Griffin*et al*., 1996; Huether *et al*., 1996; James, 1996; Morris *et al*., 1998; Bianca*et al*., 2001).

In the present study, a simple PCR-based method for trisomy detection was used where polymorphic allelic fragments were separated in polyacrylamide gel in plate V. The results of gel electrophoresis showed that trisomy 21 was detected in 100% cases by the presence of chromosome 21 in all DS subjectswith only one STR marker D21S11 even though it did not show the parental origin of chromosome 21. This is similar to the work of Shalu*et al*., (2010) were they detected trisomy21 in 86.67% cases with only two markersD21S2055 and D21S11.

The results disagree with the work of Ghosh and Dey (2003) who showed the origin of extra chromosome to be maternal in about 45% of the cases of free trisomy 21 and paternal in about 25% using two STR markers D21S11 and D21S2055 in India. These results contrast with those obtained in earlier molecular studies where paternal nondisjunction accounts for

approximately5%-6% of free trisomy 21 cases only (Antonarakis, 1991; Sherman, 1991; Ko *et al*., 1998).

Our results is also inconsistent with the work of Altaf and Irshad (2012) were trisomy 21 was detected by the presence of three additional alleles and transmission of alleles from parents to the off springs using the same D21S11 and D21S2055 markers situated on chromosome 21 at 21q21 and 21q22 respectively in Pakistan.

Rapid diagnosis becomes essential especially when the couple comes late for an antenatal diagnosis. The other method available is fluorescent in situ hybridization using uncultured cells, but this too needs proper setup and skilled personnel. Rapid diagnosis by PCR-based methods using polymorphic STR markers may reduce these difficulties. Using this method, we were able to detect trisomy in 100% cases with only one marker. Clearly, the judicious choice of a few highly polymorphic markers is very essential for trisomy detection and investigation of the parental origin of trisomy 21 (Chakravarti, 1989). This method was found to be comparable to the quantitative fluorescence technique where fluorescently labeled primer, DNA sequencer, and Genescan software are usually required for genotyping (Findlay *et al*., 1998a; Findlay *et al*., 1998b; Blake *et al*., 1999; Valero *et al*., 1999).Thus, this PCR-based technique can be applied for early prenatal diagnosis in resource-limited settings. Using a greater number of markers can further increase the reliability of the test.

The analysis byAltaf and Irshad (2012) using the microsatellite markers/ short tandem repeats as inD21S11 and D21S2055 showed that the origin of extra chromosome was maternal in about 92% of the cases of trisomy 21 and paternal in about 8% (Kava *et al*., 2004; Crkvenac -Gornik *et al*., 2007).

Age at referral of postnatal DS subjects were categorized based on age at which they are clinically diagnosed by the paediatricians. In the present study, 60% of DS were diagnosed at < 1 year and 32% between 1-2 years. This is similar to the work of Al-Harasi (2010) who found 55% of DS subjects diagnosed at > 1 year and 34.9% between 1-6 months of age.

The birth order of children with DS ranged from 1 to 5 according to Adler's theory. Overall, 22% of them were of the last birth orders. This result agrees with previous studies by Murthy *et al*.(2007) in the UAE and Dhaka by Munsi *et al*.(2014). Several studies suggest an increased risk of DS with increasing parity (Doria-Rose *et al*., 2003), but at the same time, other studies reported that there is no increased risk with increasing parity (Chan *et al*., 1998).

Some studies suggest that first borns may be at high risk of DS to older women than a later born child to women of the same age (Alfi *et al.,* 1980).This finding agreeswith the work of Stene*et al*.(1981)who found that the first-born infants were at a lower risk of DS than later born infants. Social factors affecting DS mothers are literacy, status of women, employment, general economic development, decreased fertility and adopting small family norms, adoption of family planning etc. Parental consanguinity has been in debate for a long time as one of the causes of

non-disjunction. It was observed a fourfold increase in the relative risk of DS children in closely related parents as compared to non-related parents (Alfi *et al*., 1980).

Clinical facial features were the foremost indicators of clinical suspicion of Down syndrome. Among the clinical features studied, upward slanting palpebral fissures, low set ears, flat occiput and simian crease were the most frequent feature observed (35%), which disagrees with other studies of Kava *et al*., (2004). Similarly, in our study Simian crease and hypotonia agreed with the work of Fryns, (1990) and Azman *et al.*,(2007).

There is variation in the frequency of clinical features from locality to locality. For instance, single Simian crease was present in 35% of the patients in this study; this is slightly lower than the 39% recorded in the Port Harcourt study (Otaigbe *et al*., 2012) and the 83.9% in the Brazilian study (Berteli *et al*., 2009). There were no Brush field spots identified in this study, which contrasts with a Korean study (Kim *et al*., 2002), but at variance with a Jordanian study (Kawar *et al.,* 2010) where 9% of children with DS had Brush field spots. The difference in findings may be due to the known low frequency of Brush field spots in DS individuals with dark colored races (Wallis, 1951).

Microglossia and short neckwasobserved in 33% of the evaluated children. This agrees with the work of Kava*et al*. (2004)who described similar frequency (29.9%) in a sample of DS individuals in India.The major three clinical features present in more than 50% of the cases were the flat occiput, microcephaly and hypotonia.

The observation of congenital heart defect (CHD), in more than 8% of the total cases in the present study are consistent with a study by Kava *et al*. (2004). The incidence of heart defect was encountered in 8% of this study subjects. It was interesting to note that heart diseaseswere predominant among Down syndrome children born to mothers aged more than 35 years. This is because of advanced maternal age which is seen as a major risk factor of DS. The most common defect observed wasatrioventricular septal defect (AVSD) (5%) and the second was ventricular septal defect (3%).This agrees with the work of Sharma *et al*. (2013) where atrioventricular septal defect (AVSD) was about 13 (37.142%) among 39 Down's syndrome patients, while ventricular Septal (VSD) was the most common in 26 (68.42) in an Indian population.

This result also disagrees with the work of Somasundaram and Ramkumar (2018) who observed VSD in 34.5% followed by Endocardial cushion defect in 21.8%, ASD in 20% of cases and Patent ductus arteriosus in 14.5% of cases.Our results also disagree with the work of Laursen (1976) who found VSD in 49% of 80 cases of children with CHD.

However, regarding the other clinical features, pes planus and hypothyroidism inconsistencies were noted when compared to the other studies by (Fryns, 1990; Jones, 1997; Kumar and Delatycki, 2001).

This study found that CHDs occurred in 20% of infants with DS, which is lower than almost the prevalence presented in the previous literature in India (44%-58%) (Sharma *et al*., 2013).It has been reported that the prevalence of CHD varies, depending on the presence of DS and other non-chromosomal abnormalities. For instance, the Baltimore-Washington Infant Study found AVSD in only 2.8% of non-DS cases, compared to 60.1% of DS cases. As indicated by various studies, several CHDs observed in non-chromosomal abnormalities are rare, although they are common in DS cases.

The commonest type of congenital heart defects in this study group was AVSD accounting for57.5%.This valueismuch lower than the report of Khan and Muhammad, 2012 (90.3%) in Peshawar, Pakistan and alsolower than the Libyan population with 65% isolated lesion, 80% in Guatemala (Elmagrpy *et al*., 2011) and 74% in Mexico ((Vida *et al.*, 2005).

The various reasons for this difference may include the genetic make-up of each ethnic, racial and the specific embryological mechanism (Khan and Muhammad, 2012).

Our resultsdisagree with the work of Sharma *et al*. (2013)in Indian population where ventricular septal defect (VSD) was the most common in about26 (68.42%) of the 39 Down's syndrome patients, while ventricular septal defect (AVSD) occurred in 13 (37.14%) of the cases.

One of the most important risk factors for non-disjunction of chromosome 21 is advanced maternal age.In this study, 50% of mothers under 36-40 years and 80% of mother > 40 years had their maternal ages at birth of their DS children. Occurrence of DS independent of maternal age presents an evidence for other risk factors for this syndrome for which are free trisomy 21, or

translocation or mosaic. This finding is very similar to that of Belmokhtar *et al*., (2016) where 54.5% of all Down syndrome belonged to free trisomy Down syndromeborn to mothers who were in the advanced age group (≥ 35 years).

This agrees with other report in many previous studies in different countries: India (Kava *et al*., 2004) Turkey (Alp *et al*., 2007), Malaysia (Azman *et al*., 2007), England and Wales (Mutton *et al*., 1996), Jordan (Amayreh *et al*., 2012),Saudi Arabia (Qahtani *et al*., 2011), Tunisia (Chaabouni *et al*., 1999), and Dubai (Murthy *et al*., 2007).

Chromosomal non-disjunction is a random event that occurs more frequently as women get older. However, since it can occur at any time, children with trisomy 21 can be born to women of all ages. In fact, because most pregnancies occur in younger women, approximately 80% of all babies with trisomy 21 are born to women under the age of 35 (Holmes, 1978). In the present study, 60% had maternal age of 35 years. The chromosomal profiles of Down syndrome cases having maternal age ≥35 years showed 80% non-disjunction.This disagrees with the work of Kaur and Singh,(2010) who reported that 76.6% of Down syndrome are born to women less than 30 years of age in India. Thus, offering the evidence that advanced maternal age increases risk for a non-disjunctional event in the ovum (Erickson, 1978).

In our study population, mean maternal age at birth of the affected child was 35.66 ± 8.53 years. It was significantly higher than mothers of non-trisomic children (31.28 ± 5.96 years).This result is similar with the study of El‑Gilany *et al*. (2011) in Egypt, where the mean maternal age was

36.8 years, and the study of Jaouad *et al.*(2010) in Morocco, the mean maternal age was 35.39 years. Also similar with the result found by Verma *et al*. (1991) in Libya was 35.62 years. It was reported in many previous studies in different countries: India (Kava *et al*., 2004), Turkey (Alp *et al*., 2007), Malaysia (Azman *et al*., 2007), England and Wales (Mutton *et al*., 1996), Jordan (Amayreh *et al*., 2012), Saudi Arabia (Qahatani *et al*., 2011), Tunisia (Chaabouni *et al*., 1999) and Dubai (Murthy *et al*., 2007).

Out of the 16 Down syndrome patients, 64% were born to mothers older than 35 years of age. This clearly indicated that maternal age was a major contributing risk factor in a significant proportion of cases in this population. A sudden increment was noted in the percentage of 60% cases between the group of mothers (31-35 years) and the difference was increasedby 80% as the maternal age approaches >40 years. This data is consistent with the exponential increment of 88% noted by Epstein (1995).

The existence of a paternal age effect on Down syndrome is controversial. In our study paternalagehas no effectonDownsyndromebutonlyin discrimination. This disagrees with the work of Sartorelli*et al*. (2001) who studied a small population of men and reported a higher frequency of sperm chromosome abnormalities in older men

It is well known that aneuploidy can have major detrimental health consequences when it occurs in either germinal or somatic cells. Germinal aneuploidies, a major cause of pregnancy loss,

aneuploid births and developmental defects, (Wyrobek *et al.*, 2000) are thought to arise de novo, through meiotic errors in germ cells of either parents, or mitotically shortly after fertilization. Both age-dependent and age-independent factors appear to be operating simultaneously. It could be due to age-dependent decay in the spindle fibers or their components, a failure in nucleolar breakdown or an accumulation of the effects of radiation, hormonal imbalances and infection (Chandley, 1985). On the other hand, clinical and experimental studies have shown that age-independent DNA hypomethylation is associated with chromosomal instability and abnormal segregation. Based on this, Christman *et al.* (1993) suggested a link between dietary folate and methyl deficiency in vivo and DNA hypomethylation (Christman*et al.,* 1993). Based on these cellular observations, James *et al.* (1999) and Hobbs *et al.*(2000) have postulated a link between abnormal folate metabolism and mutation of the methylenetetrahydrofolate reductase gene, hence as a risk factor for nondisjunction and Down syndrome in younger (< 35 years) mothers.

Many other studies had shown increased number of DS babies born to the young mothers, like the study of Kava *et al*., (2004)in India, the maternal age at birth of affected children was 26.8 years. Other study in the same country reported a mean of 24.95 years (Sheth *et al*., 2007). For older mothers, the maternal age effect may be due to differential selection and accumulation of trisomy 21 oocytes in the ovarian reserve of older women (Hulten, 2008). For younger mothers, the mechanism behind the nondisjunction is not well understood. One of the reasons couldbe that the ovaries of young women are biologically older than their chronological age, which may lead to increased incidence of nondisjunction (Schufp *et al*., 1994).

In assessing for the association of maternal age with the occurrence of congenital heart defects in these DS subjects. The incidence of CHD in DS subjects was increased in both maternal and paternal ages of > 40 years. This contradicts the findings by Chehab*et al.* (2007) who documented lesser occurrence of CHD in DS patients with maternal age above 32 years. Our result is inconsistent with the work of Animasahun *et al.* (2016) who found incidence of CHD in DS subjects was decreasing from maternal age 36 years and above.

Women of younger age groups were found to have children with Down syndrome which is similar to study by Bertelli *et al*. (2009). This may probably be due to occurrence of unbalanced translocation in these mothers and possibly by other environmental risk factors such as cigarette smoking, exposure to chemicals, toxins, ionizing radiation, and folate deficiency. The effect of advanced maternal age as a risk factor for having a child with Down syndrome is limited to nondisjunction errors that occur in the ovum (Mohammed, 2013).

CHD is the most common cause for long term morbidity and mortality in DS. CHD was found to be the more in female DS that male DS. This result disagrees with the work of Somasundaram and Ramkumar (2018) where they found 40-50% of male with CHD than female DS subjects.

The most common type of DS was free trisomy with highest occurrence in patients > 40 years. This is similar to the work of Sotonica *et al*. (2016) who observed free trisomy 21 among DS children in Bosnia and Herzegovina, and it is also the most common in both mother and father's age's group from 30 to 39 years old.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The current investigation is the first comprehensive study of Down syndrome in Kano. The cytogenetic study includes the karyotypic findingsin 16Kano children with DS who were diagnosed at the Murtala Muhammad Specialist and Hasiya Bayero Paediatric Hospitals. The data show that 100% of the children with DS have a free trisomy 21, whiletranslocation and mosaicism trisomies have 0%. These results are similar to the findings of other published studies. Out of the 16 liveborn DS children with a free trisomy 21 there were 9 males and 7 females resulting in a sex ratio of 1.3:1. The skewed sex ratio in Down syndrome has also been reported from other studies, however, the underlying mechanism is still unexplained.The molecular genetic study determined the sensitivity of DNA diagnosis of DS using PCR and STR marker for detection of trisomy 21.Out of the 35 informative DS families,16 cases (100%) were of mitotic non-disjunction.

Down syndrome among Kano metropolis population was more frequent in males (56.2%) than females (43.75%).

Clinical features and birth order of DS patients showed statistically significant differences with the control. The commonest type of CHD in DS subjects was AVSD followed by ASD.

One of the most important risk factors for non-disjunction of chromosome 21 is advanced maternal age. The current study in Kano State demonstrated a very strong association of advanced maternal age with the birth of a DS child. The case control study showed that the mean maternal age of the mothers of DS children was 35.66 years which is significantly higher than the maternal age of the control mothers with 31.28 years. However, the maternal age-related risk factor alone cannot explain the unusually high DS birth prevalence in Kano State, Nigeria.

A cluster analysis of the DS associated risk factors with the occurrence of CHD showed no statistically significant differences. Association of free trisomy 21 and parental age showed statistically significant differences. The highest occurrence was in DS maternal and paternal ages > 40 years. The mean maternal and paternal ages were 35.6±8.53 and 46.34±11.7 respectively.

The most prevalent clinical features seen in children with DS in this study were upward slanting fissures, low set ears, macroglossia, flat occiput, simian crease, short neck, hypotoniaand CHD. Hypothyroidism and pes planus cases were not common in our present study.

In this present study CHDs occurred in 30% of infants with DS.Ventricular septal defect(57%) was the commonest cardiac lesion in DS followed by atrial septal defect (43%) in our study.

6.2 Conclusion

It can be concluded that free trisomy of chromosome 21, resulting from a chromosomal nondisjunction is the most frequent causethan translocation and mosaic DS. The study showed interesting single pattern in the frequency of Down syndrome in Kano. The knowledgeof the type of trisomy 21 and status of the parent is important to estimate the risk of recurrence in future pregnancies. This information will assist with advances in prenatal diagnosisthat canassist parents in decision making that may reduce the burden of Down syndrome births.

It can be concluded that sixty (60%) of DS children born in Kano metropolis are diagnosed cytogenetically in less than one year. This is a very good indicator that the paediatricians are aware of the clinical phenotype and initiate cytogenetic analysis for confirmation.

The PCR-based DNA diagnostic method using STR was found to be sensitive, reproducible, and efficient, not only for diagnosis of trisomy 21, but also for tracing allelic transmission from parents to the offspring. This method can also be employed in the diagnosis of trisomy 13 and 18. Furthermore, it may be useful for prenatal diagnosis using fetal DNA from maternal blood, and for pre-implantation genetic diagnosis and prenatal counseling.

This study concludes that children with free trisomy of chromosome 21 are more frequently born to mothers older than 35 years of ageand that significant risk levels for Down syndrome are in advanced maternal age categories. However, paternal age appears not to affect the frequency of giving birth to a child with trisomy 21.

This study concludes that mean maternal age at birth of the affected children was 35.66 ± 8.53 years.Also, it concludes that children with DS are commonly born in the last-born order.

This study also show that paternal age also has no effect as a risk factor of giving birth to DS patient.

Frequency distribution of sex and birth order amongst clinically diagnosed DS in this study was found to be higher in DS without CHD.

The study showed strong relationship in the frequency of free trisomy 21 type in DS children and its association with maternal and paternal age in Kano state.

6.3 Recommendation

1. Early diagnosis, and a proper screening for high association with systemic anomalies, should be undertaken among the Down syndrome patients in this population.
2. A larger sample size study should be done to ascertain regional and seasonal differences in the birth prevalence of DS in Nigeria.
3. Mothers age ≥ 35 years should be counselled on possible risk of having children with DS.
4. Adults with DS should also be followed up in appropriate specialty clinic.
5. Further molecular studies should be done to determine the parental origin of extra chromosome 21 of DS.
6. The frequency of other type of DS and its association with maternal and paternal ages should be determine.
7. Consanguinity marriages amongst DS parents should be determined as a risk factor of DS.

6.4 Contribution to Knowledge

i. A baseline data was obtained for DS patients of Kano State, Nigeria using cytogenetic analysis to determine the different types of DS. Based on the data model was developed discriminating normal and abnormal number of chromosomes. The use ofkaryotyping free trisomy 21for both sexes is 56.25% (males 47, XY, +21) and 43.75%(females 47, XX, +21) respectively and was the most frequent with the accuracy of 100% achieved.

ii. This study confirmed the clinical disease of DS to be reliable based on certain clinical features.

iii. The relationship between advanced maternal age of DS mothers and their children was established as a risk factor of trisomy 21(Chi-square value= 8.43, DF=4, P = 0.077). Furthermore, maternal AUC= 0.67, p=0.025) and paternal ages (AUC= 0.62, p= 0.025) at the time of birth DS patient was established.

iv.This study also established sex ratio link between male and female DS patients with more dominance ration in male than female (Male: Female=1.3.1) in Kano, Nigerians.

v. Trisomy 21 was identified with the presence of three distinct alleles in children with DS and was confirmed molecularly using PCR- based detection analysis and demonstrate the usefulness of highly informative microsatellite markers for the study of nondisjunction in Down syndrome.

vi. The mean maternal and paternal ages based on free trisomy 21 type of DS children was established ((Maternal age= 35.6± 8.53, Paternal age= 46.34± 11.7, $p<0.0001$)respectively.

vii. Women of younger age (<25 years) groups were found to have children with Down syndrome.

viii. The percentage of CHD based on type between male and female patients was established (AVSD= 4% and ASD= 2%).

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APPENDIX I

CONSENT TO PARTICIPATE IN OUR RESEARCH

A research is being conducted of cytogenetic analysis of children with Down syndrome. We want to seek for your permission to enroll your child to participate in this study. You have the right to participate or not. This will not interfere with any form of care being giving to your child and it is at no cost to you. The information obtained is strictly confidential.

If you have any question or concerns about the research, please feel free to contact MUHAMMAD, Abdulhakeem Miko Department of Human Anatomy, Faculty of Basic Medical Sciences, ABU, Zaria, 08035682913, abdulhakeemmiko@gmail.com.

Purpose of the Study: Cytogenetic and Molecular Genetic Studiesof Children with Down syndrome and its associated risk factors in Kano State.

1. **Procedures:** The data collection will involve collecting information regarding bio data, here the participant will be ask to provide some information relevant to his bio data. In the second phase of the study blood sample will be taken from your child for a cytogenetic analysis which will be done within 72 hours and molecular analysis using STR markers.
2. **Potential Risk and Discomfort:** There is no associated risk with this procedure and the only discomfort may be the time you will sacrifice while taken the blood.
3. **Potential Benefits to Participant and/or to society:** This research may be of potential benefit to the participant and/or society in the following way:

   Opportunity to know your patterns of your chromosomes in relation to congenital disorders and others.
4. **Payment for Participation:** Incentive and refreshment will not be offered to the participant after participation.

5. **Confidentiality:** Every effort will be made to ensure confidentiality of any identify information that is obtained in connection with this study. The variables and information collected will only be used for the aims and objectives of the study as well as scientific publications. I assure you that your pictures and other information will be kept in strict confidence.
6. **Participation and Withdrawal:** You can choose whether to be in this study or not. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. You may exercise the option of removing your data from the study. You may also refuse to answer any questions you don't want to answer and remain in the study. The researcher may withdraw you from this research if circumstances arise that warrant doing so.
7. **Right of Research Participants:** You may withdraw you consent at any time and discontinuous participation without penalty. You are waiving any legal claims, right or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through Committee on ethics from Kano State Hospital Management Board, Kano State. If you have questions regarding your right as a research participant contact; Committee on ethics, Kano State Hospital Management Board, Kano., Tel:
8. **Signature of Research Participants/ Legal Representative:** I have read the information provided for the study titled. Study of Cytogenetic Analysis of Children with Down syndrome as describe herein. My questions have been answered to mysatisfaction, and I agree to participate in this study. I have been given a copy of this form

_____ _____

Name and signature of the participant Name and signature of thewitness

OR

_____

Thumb print of the participantThumb print of the witness

APPENDIX II

A DESIGN OF INFORMED CONSENT FOR DOWN SYNDROME FAMILY

HOSPITAL NO: ----------
NAME: ----------
AGE/DOB: ----------
SEX: ----------
NATIONALITY----------

Ward/Department: ---------- Unit---------

WRITING EXPRESSED GENETIC TESTING CONSENT FORM

(A) I ---------- Age ---------- resident of ---------- hereby give this written consent/permission for the genetic testing for my child----------.

Relative ----------

Relationship----------

I had been given adequate information about effects. Potential harms and benefits of genetic testing, about its limitations, possibilities of informative results and chances of exact prediction.

I had been informed that the results will be kept strictly confidential and should not be disclosed to anybody without my permission.

Patient/Relative ----------

Signature ---------- or Thumb print----------

Date ---------- Time----------

APPENDIX III

CYTOGENETIC REQUEST FORM FOR THE PATIENT WITH CLINICAL INFORMATION

Department of Human Anatomy

Faculty of Basic Medical Sciences,

Ahmadu Bello University, Zaria Lab No: ----------

FORM FOR CONSTITUTIONAL KARYOTYPE AND CYTOGENETIC ANALYSIS

Hospital	Ref. Doctor's Name:
Hospital No:	Address:
Name of patient:	Signature:
Age: Sex:	Tel No:
Nationality:	Date:

Request:

Type of specimen (specify)

Blood culture □ Bone Marrow Aspirate □

Peripheral blood Lymphocyte □ Others (specify)--------- -

<table>
<tr><td colspan="2">Clinical information (symptoms, signs & diagnosis)

Whether the patient is on any drug or treatment</td></tr>
<tr><td>Previous Report: Yes/No</td><td>Specimen Collection Time& Date</td></tr>
<tr><td>Specimen Receiving (Cytogenetic Laboratory)

Time: Date:</td><td>Specimen status: Good □ Insuffiecient □

Clotted □

Haemolysed □ other (specify)---------</td></tr>
<tr><td colspan="2">Cytogenetic findings:

Signature&Stamp

Technician’s Name & Signature Head of Cytogenetic Section</td></tr>
</table>

APPENDIX IV

CASE-CONTROL-STUDY

Research Location……

Research Number……

(A) Biodata

(I) Name…… Hospital No……...

(ii) Age …… Sex: Male…… Female……

(iii) Tribe / Ethnic group…… Birth order……

(iv) Age at diagnosis……. Birth…… < 6months……...1 yr.…… <1yrof age……

(B) Family History

(i) Mothers age……

(ii) Mothers parity……

(iii) Fathers age…….

(iv) Any child in the family with similar illness Yes……. No…….

If yes is an older sibling…… Young sibling……

GSM Number……

Contact Address……

(C) Clinical Features

a. Head & facial features	Yes	No
Upward slanting of eyes	……	…….
Low set ears	……..……..	
Microglossia (protruded tongue) ……	……..	
Short neck	--------	……
Flat occiput	..……	…….
Abnormal fingers	……. …….	
Abnormal size of head (small, large) ……..…….	……..	

(B) Hands

Abnormal fingers	……	…….
Simian crease (single palmer crease)	…….	…….

(C) General features

Hypotonia	……	……

Development retardation ……. …….

(If yes specify ……)

Pes planus …… ……

Sydney line ……. ……..

Congenital anomalies ……. …….

(If yes specify……...)

Associated medical condition ……. ……..

Feature of hypothyroidism ……. ..……

APPENDIX V

ABU ETHICAL CLEARANCE I

Committee on Use of Human Subjects for Research

Directorate of Academic Planning & Monitoring

Ahmadu Bello University, Zaria

Chairman: Prof I. H. Nock, B.Sc,M.Sc (ABU), PhD (NewDelhi) 08065425450 |

Appl No.: ABUCUHSR/2020//001
Approval No: ABUCUHSR/2020/001

13th March, 2020

Dr. S.A. Musa,
Department of Human Anatomy,
Faculty of Basic Medical Science,
Ahmadu Bello University,
Zaria

Dear Sir,

PROVISIONAL APPROVAL OF RESEARCH TITLED 'ASSOCIATED RISK FACTORS, A CYTOGENETIC AND MOLECULAR GENETIC STUDIES OF CHILDREN WITH DOWN SYNDROME IN KANO STATE, NIGERIA'

This is to convey the provisional approval of the ABUCUHSR to you for the aforestated study domiciled in the Department of Human Anatomy. The approval is predicated on the assumption that you shall maintain and cater for the study subjects as indicated in your application.

Monitoring of the Research by spot checks, invitations, interactions with the subjects by any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

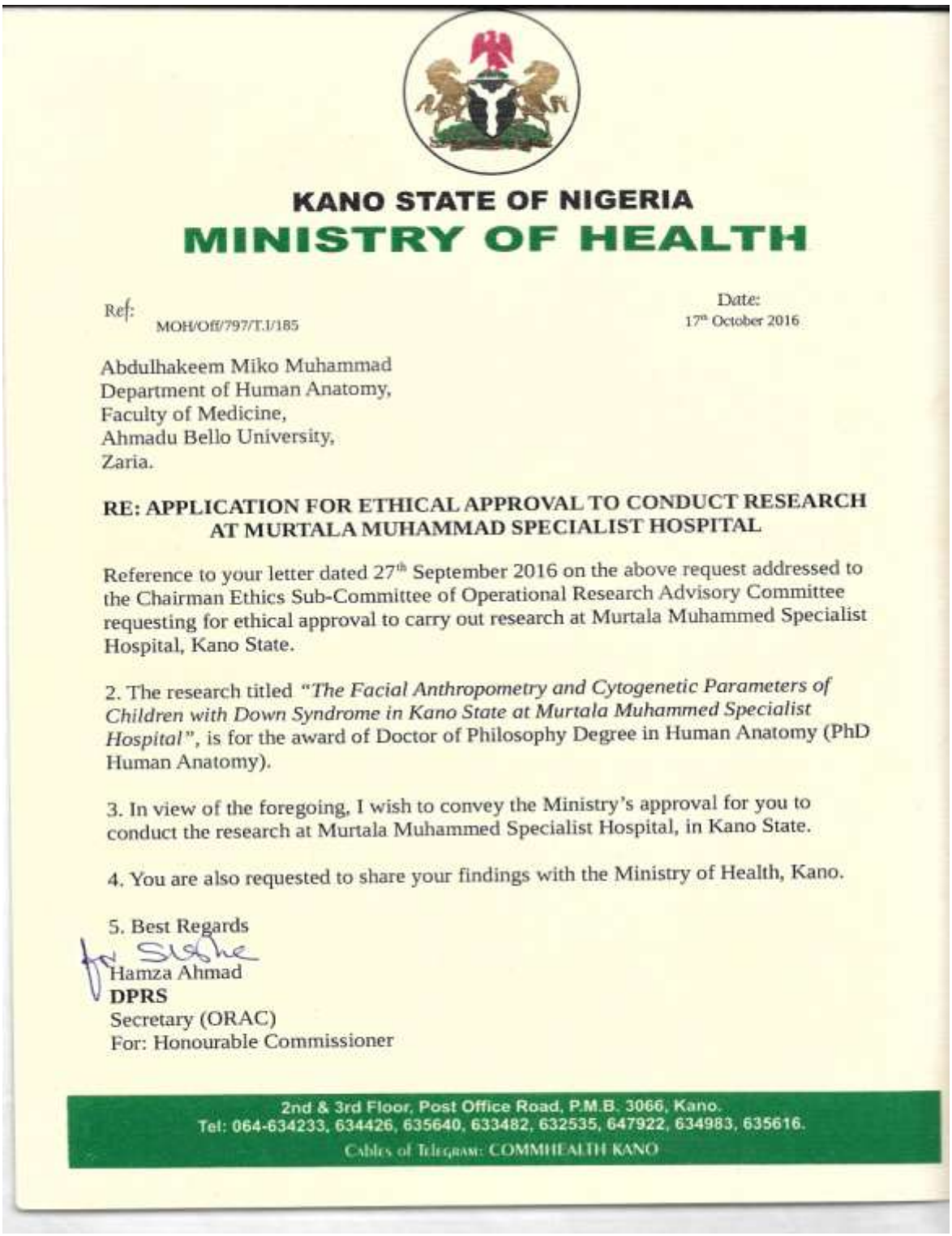
The said approval shall be posted on the ABUCUHSR Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

Prof. I.H. Nock
Chairman, ABUCUHSR

Cc. Director, DAPM
" Director, IAIICT
" Dean, Faculty of Basic Medical Sciences
" Head, Department of Human Anatomy

APPENDIX VI

ETHICAL CERTIFICATE II



KANO STATE OF NIGERIA

MINISTRY OF HEALTH

Ref: MOH/Off/797/T.I/185

Date: 17th October 2016

Abdulhakeem Miko Muhammad
Department of Human Anatomy,
Faculty of Medicine,
Ahmadu Bello University,
Zaria.

RE: APPLICATION FOR ETHICAL APPROVAL TO CONDUCT RESEARCH AT MURTALA MUHAMMAD SPECIALIST HOSPITAL

Reference to your letter dated 27th September 2016 on the above request addressed to the Chairman Ethics Sub-Committee of Operational Research Advisory Committee requesting for ethical approval to carry out research at Murtala Muhammed Specialist Hospital, Kano State.

2. The research titled *"The Facial Anthropometry and Cytogenetic Parameters of Children with Down Syndrome in Kano State at Murtala Muhammed Specialist Hospital"*, is for the award of Doctor of Philosophy Degree in Human Anatomy (PhD Human Anatomy).

3. In view of the foregoing, I wish to convey the Ministry's approval for you to conduct the research at Murtala Muhammed Specialist Hospital, in Kano State.

4. You are also requested to share your findings with the Ministry of Health, Kano.

5. Best Regards

for Shehe
Hamza Ahmad
DPRS
Secretary (ORAC)
For: Honourable Commissioner

2nd & 3rd Floor, Post Office Road, P.M.B. 3066, Kano.
Tel: 064-634233, 634426, 635640, 633482, 632535, 647922, 634983, 635616.
Cables of Telegram: COMMHEALTH KANO

APPENDIX VII

ETHICAL CERTIFICATE III

Kano State Hospitals Management Board

MURTALA MUHAMMAD SPECIALIST HOSPITAL

KANO-ZONE 6

Telephone/Fax: 064-636792
064-636793

In case of Reply Please Quote Reference No MMSHZ/0324/III/167

P.M.B. 3200
KANO-NIGERIA

DR. ABDULHAKEEM
MIKO MUHAMMAD

TO WHOM IT MAY CONCERN
RE: APPROVAL TO CONDUCT RESEARCH

I am directed to introduce the aforementioned Doctor who is to conduct a research as per attached ethical clearance at this facility.

Please give him/her the necessary information may require during the research.

Best regards.

10 DEC 2016

NASIRU SABO ABUBAKAR
FOR: CHIEF MEDICAL DIRECTOR.

APPENDIX VIII

TRAINING CERTIFICATE

WEST BENGAL UNIVERSITY OF TECHNOLOGY
BF-142, SALTLAKE, SECTOR-I, KOLKATA-700 064
Web Site : www.wbut.ac.in

Prof. Subrata Kumar Dey
Professor, Department of Biotechnology
School of Biotechnology
&
Biological Sciences

Date: 12.07.2018

TO WHOM IT MAY CONCERN

This is to certify that Mr. Abdulhakeem Muhammad Miko, Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria, Nigeria has received training in cytogenetics and molecular genetics ,including hands on training and wet sample processing starting from lymphocyte culture setup, harvesting, slide making, banding, karyotyping, DNA isolation, Gel electrophoresis from 25th June- 12th July, 2018 in our Centre for Genetics Studies at Maulana Abul Kalam Azad University of Technology (Formerly WBUT), Kolkata, India.

Prof. Subrata Kumar Dey

Professor of Biotechnology &

Former Pro-VC

Prof. S. K. Dey
Department of Biotechnology
Maulana Abul Kalam Azad University
of Technology, West Bengal
Formerly Known as West Bengal University of Technology)
BF-142, Sector-I, Salt Lake, Kolkata-64

APPENDIX IX

KARYOGRAMS FOR DOWN SYNDROME PATIENTS

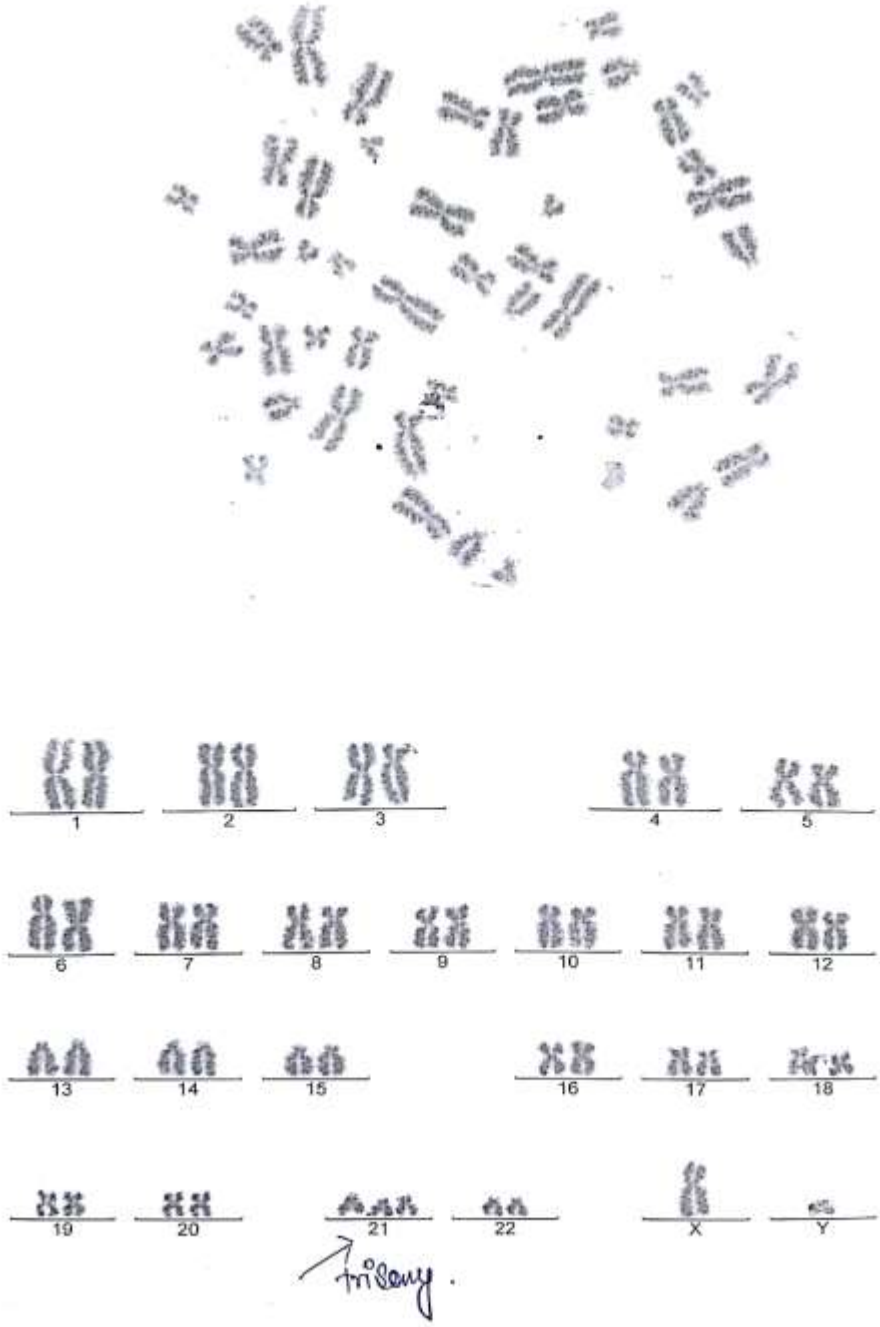


Plate I: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

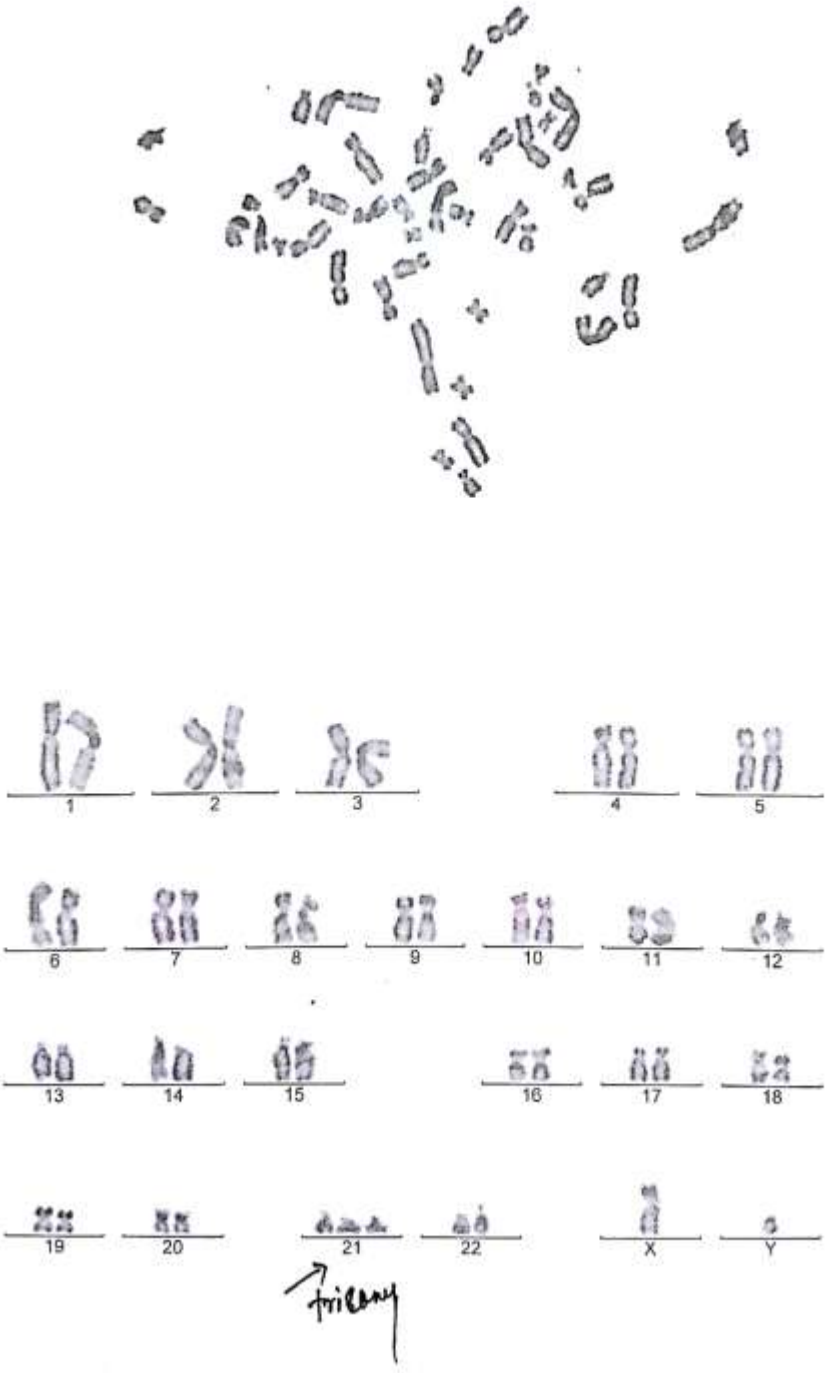


Plate II: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

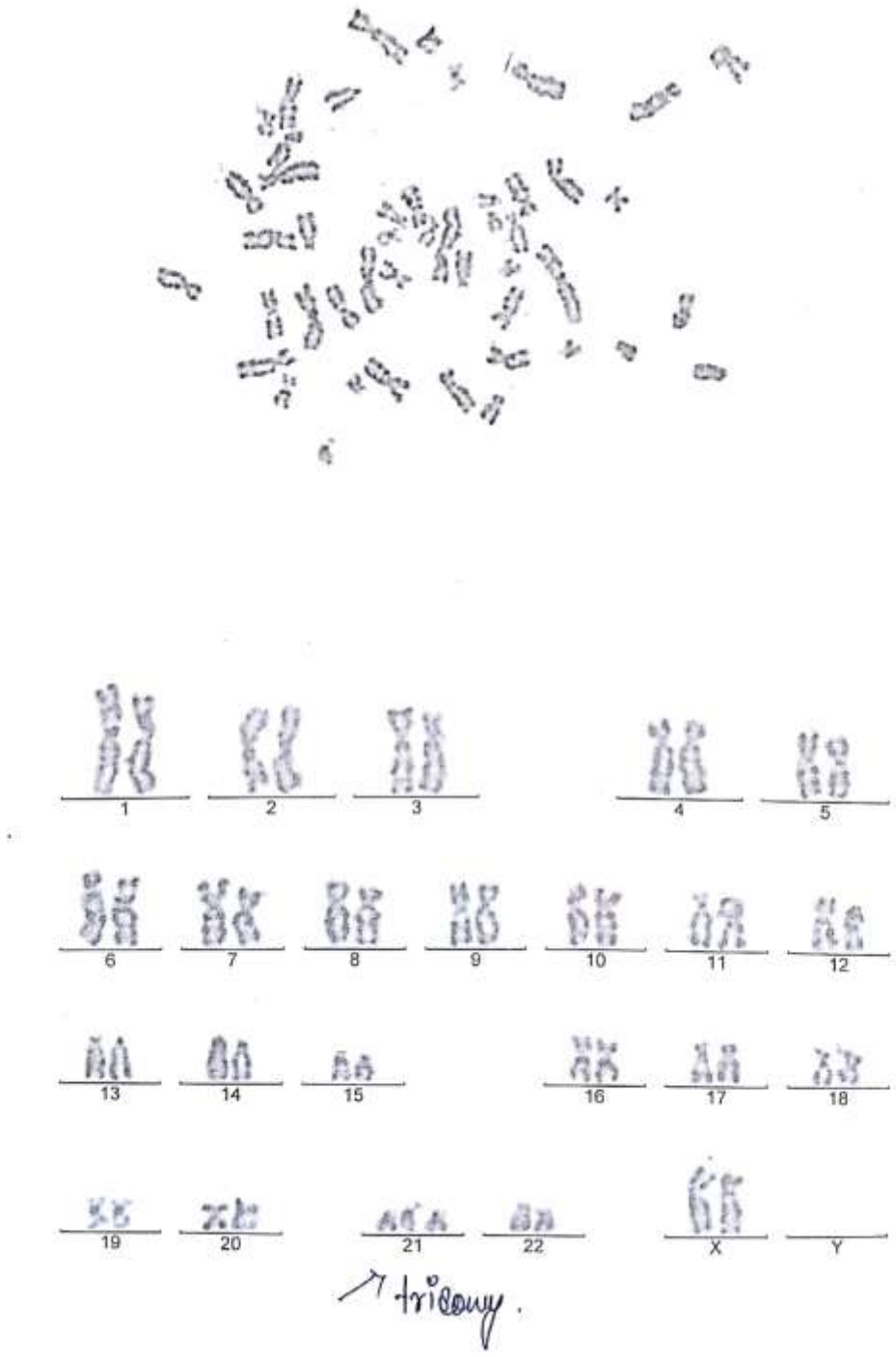


Plate III: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21

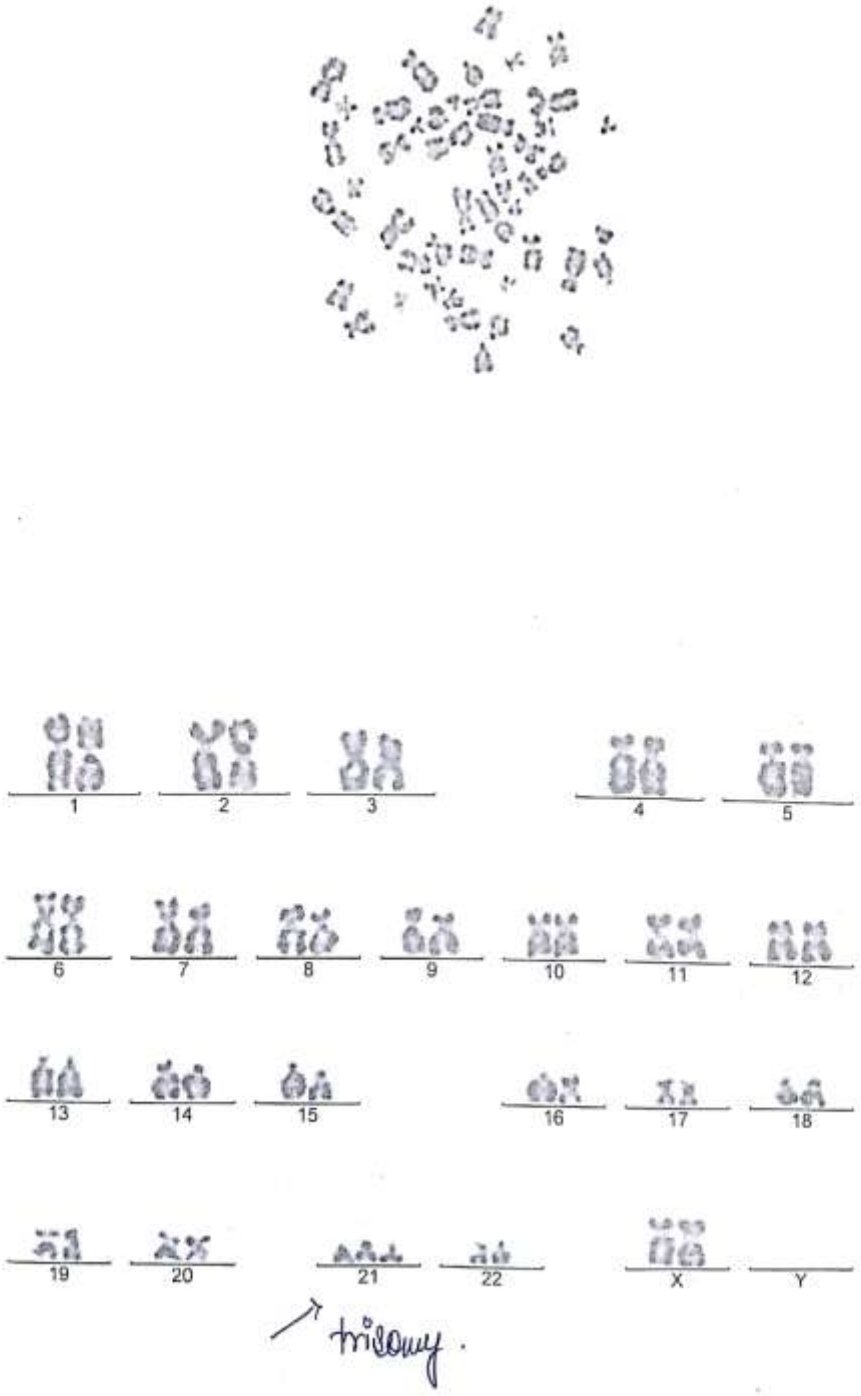


Plate IV: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21

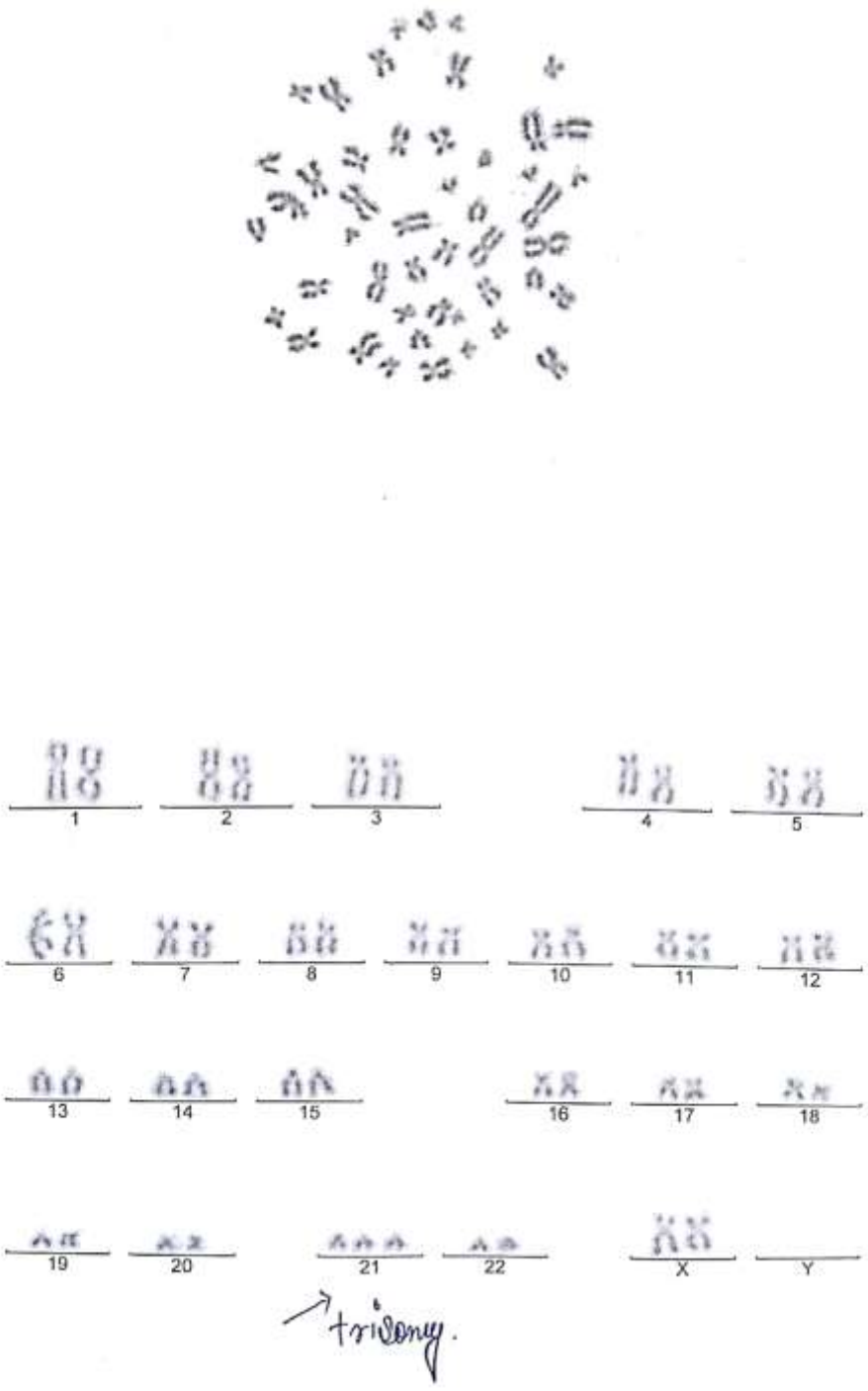


Plate V: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21

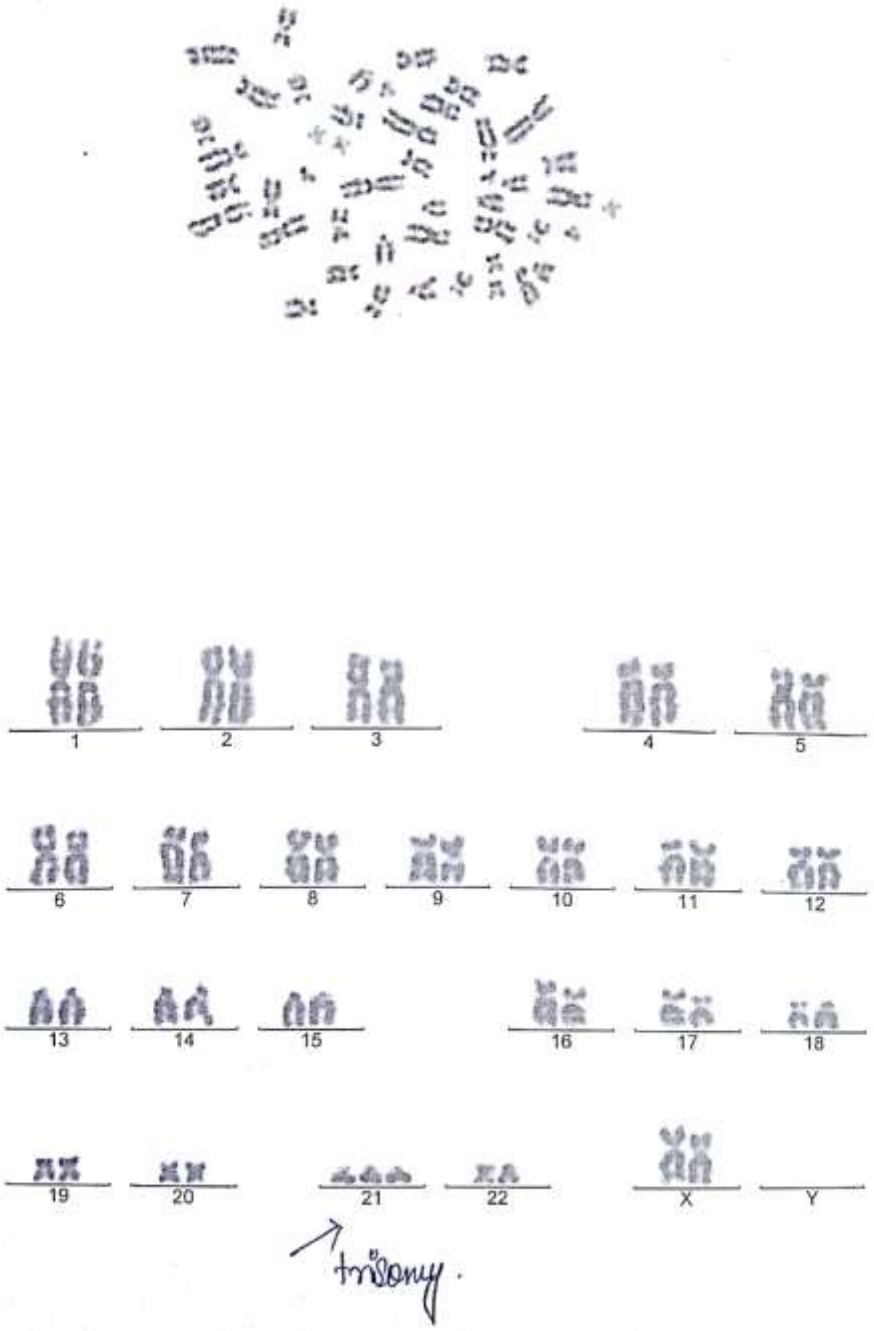
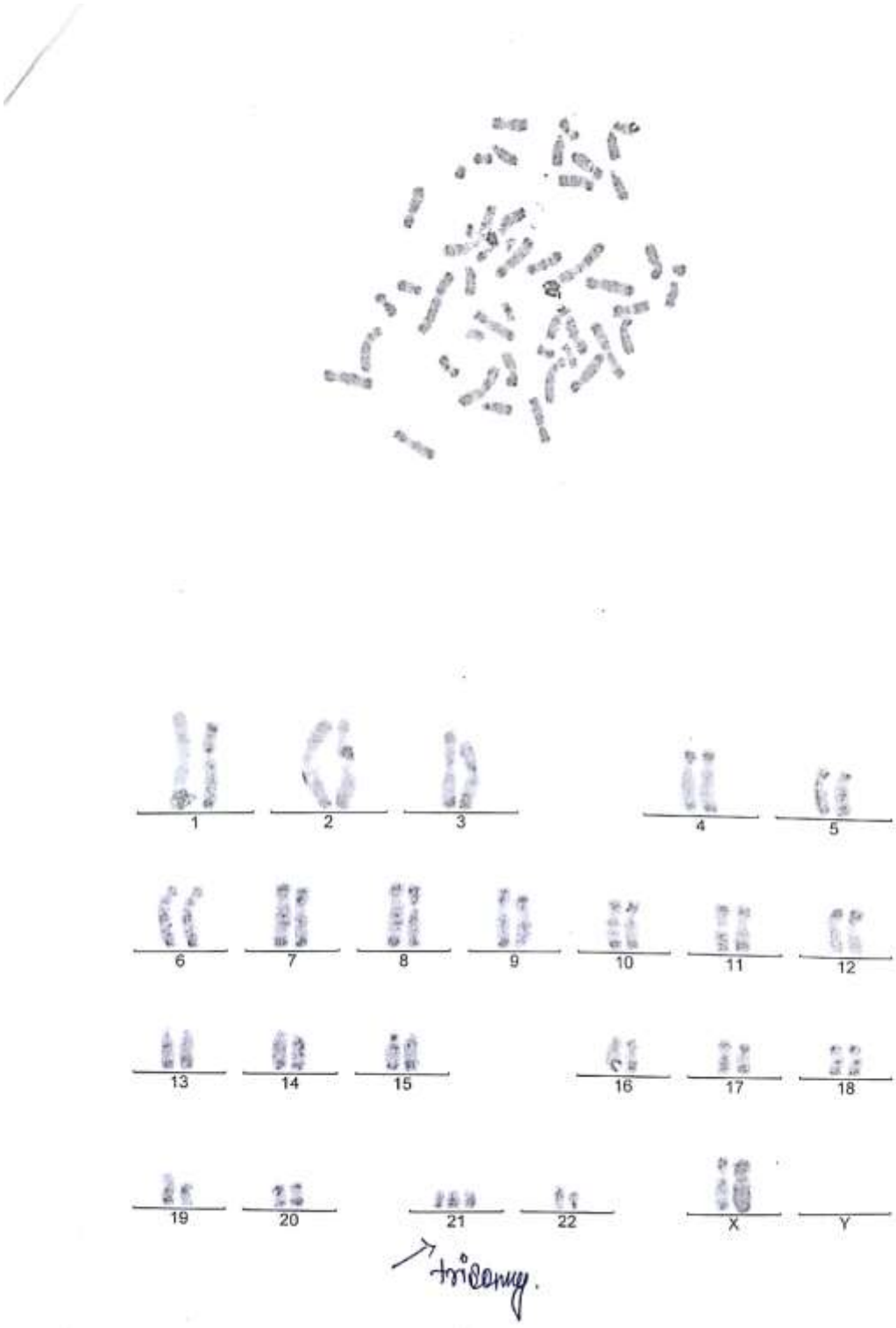


Plate VI: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21



PlateVII: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21

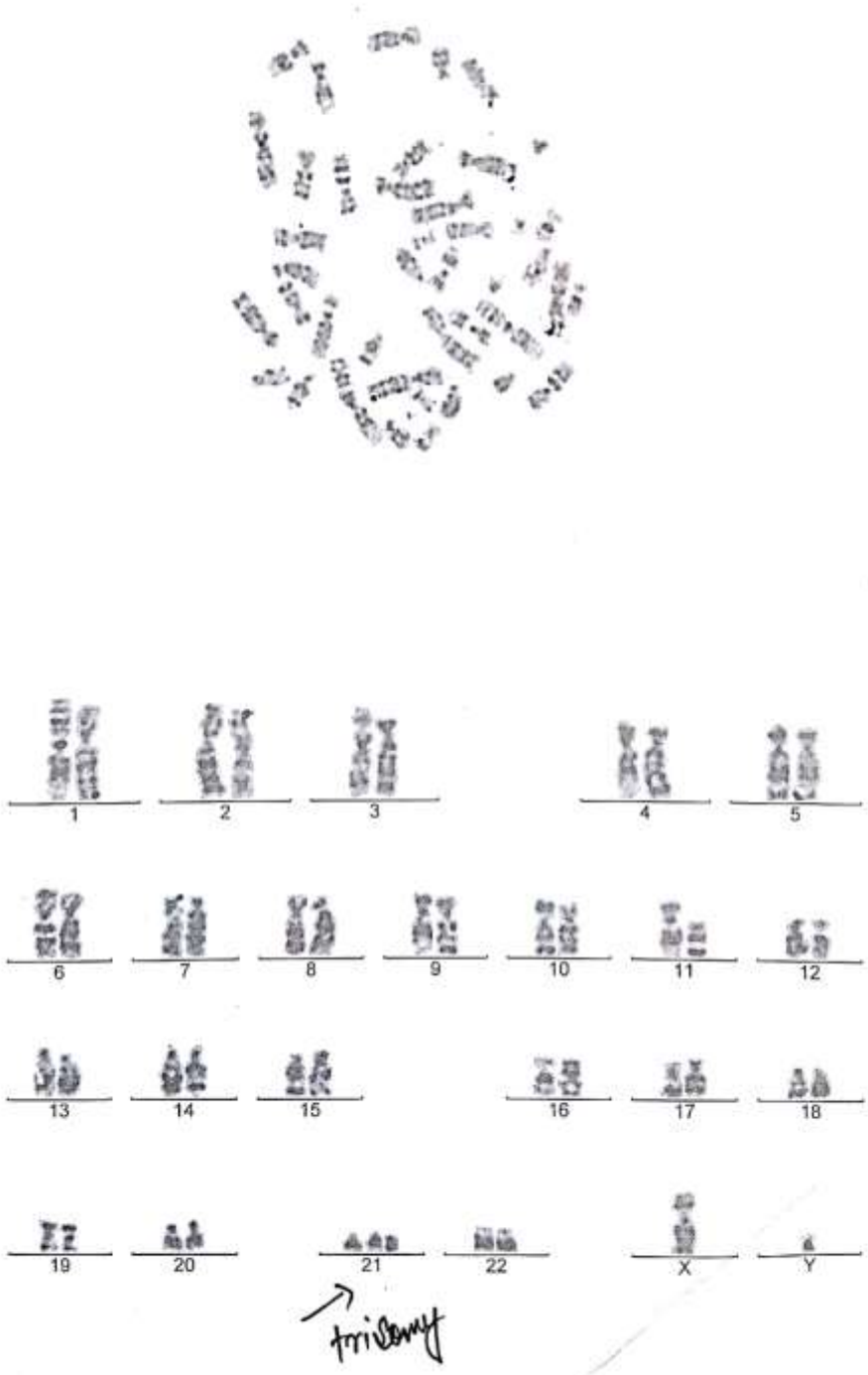


Plate VIII: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

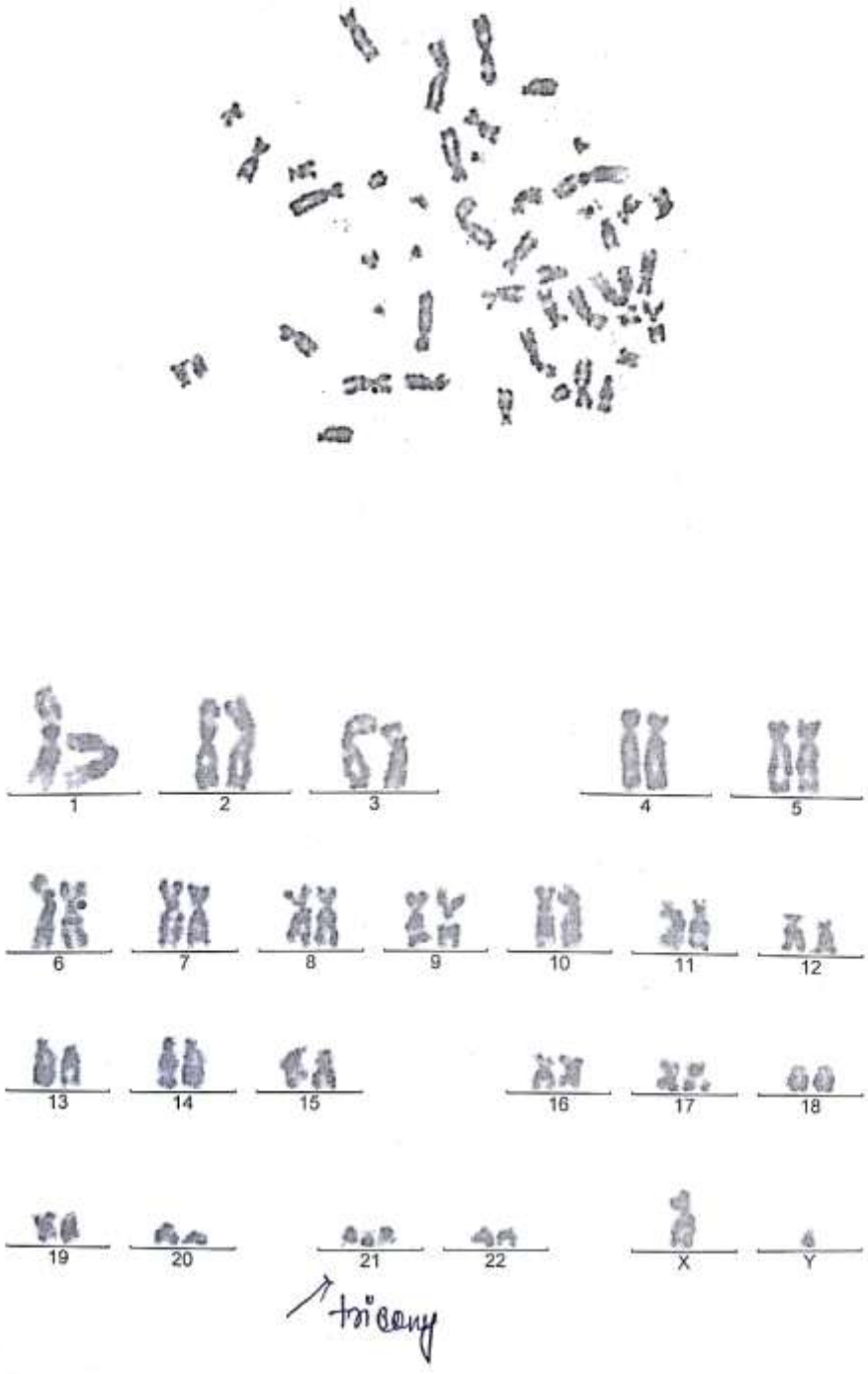


Plate IX: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

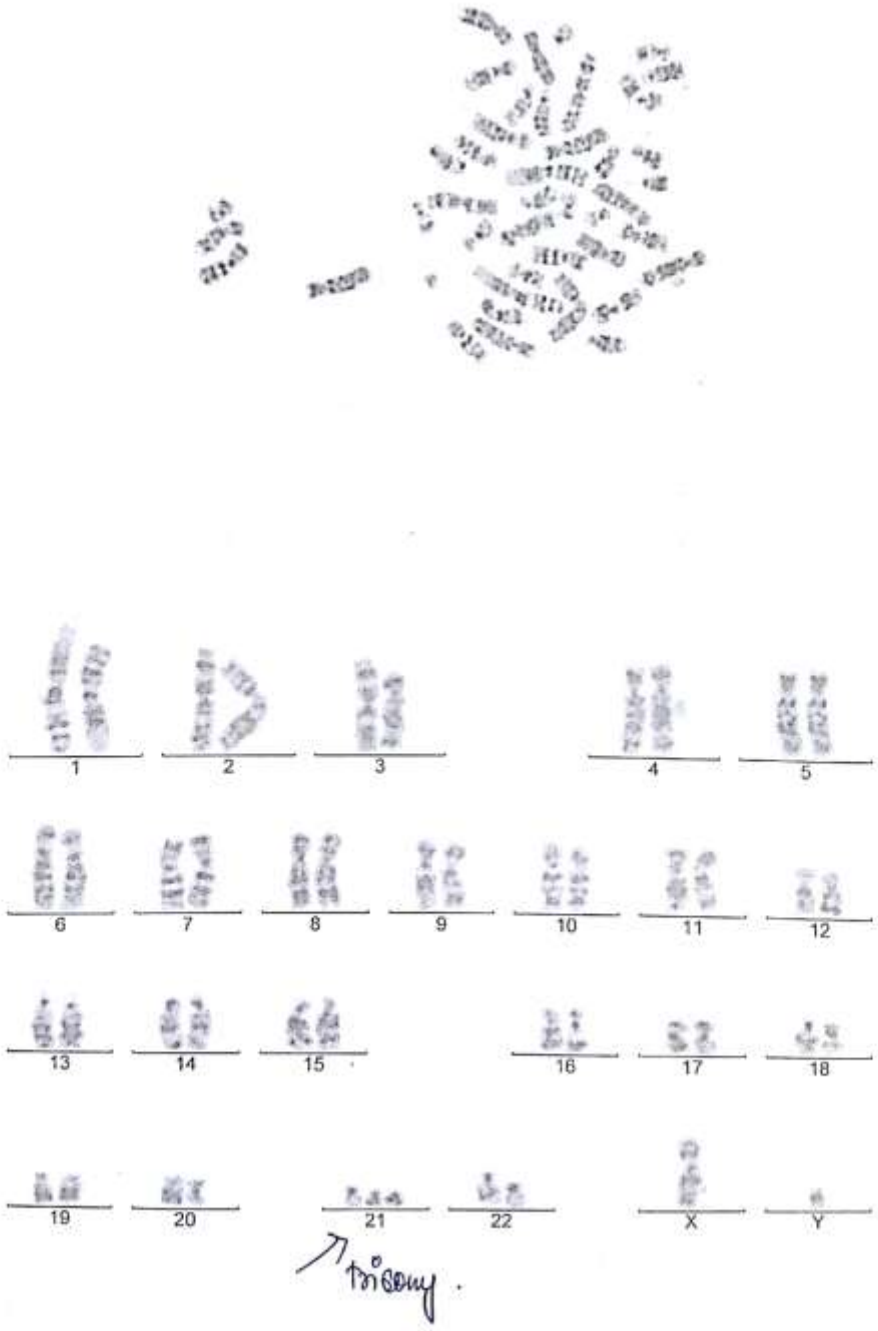


Plate X: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

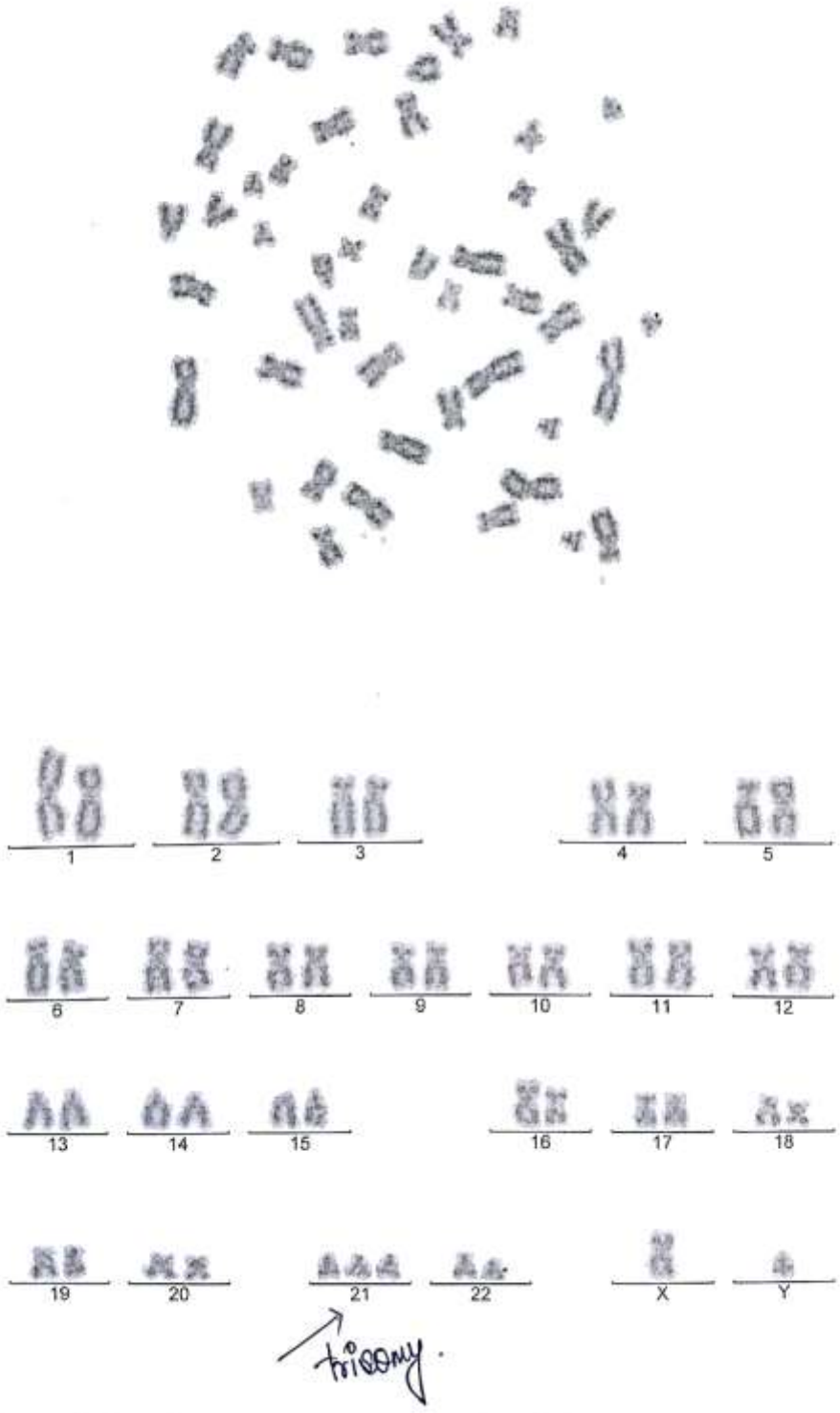


Plate XI: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

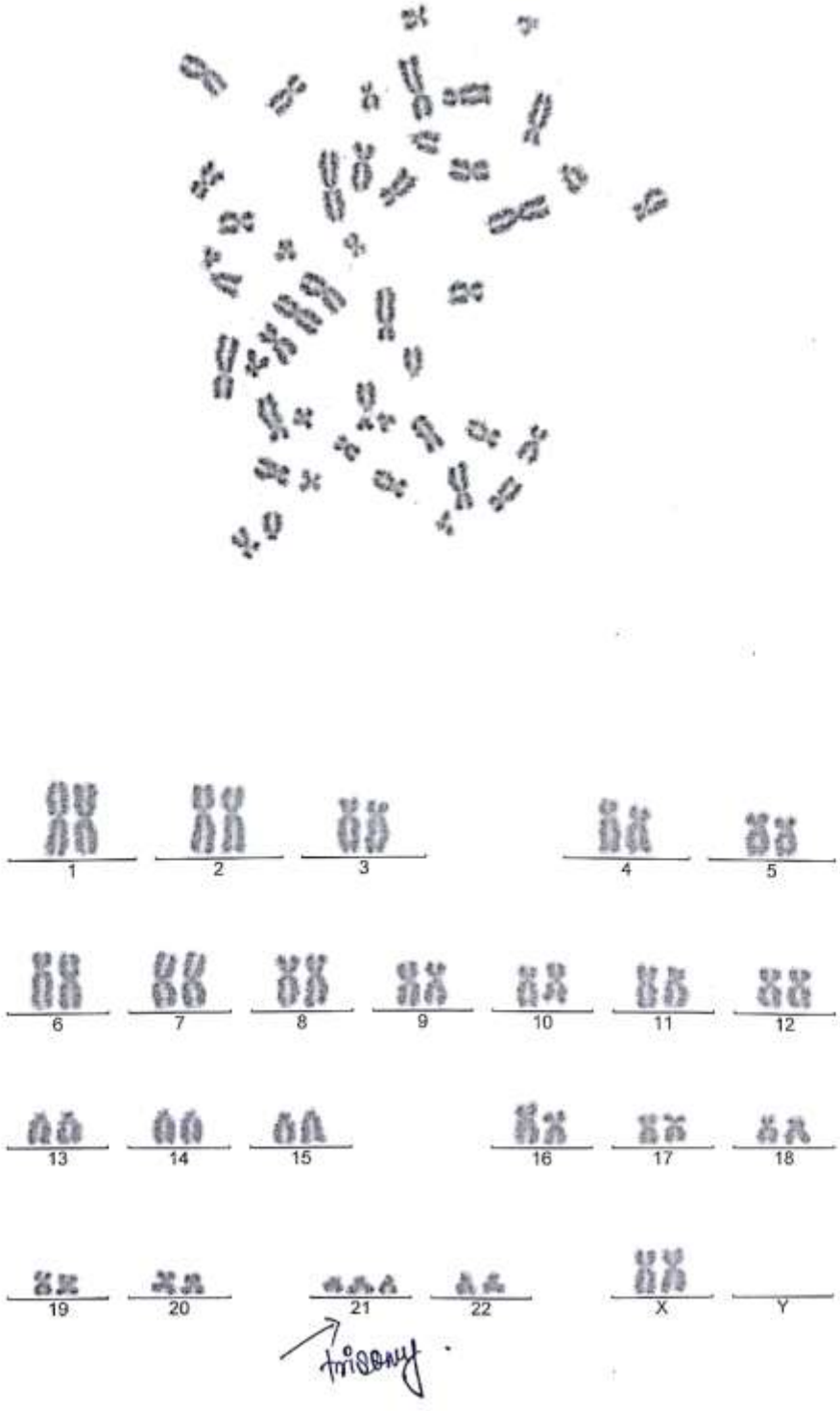


Plate XII: A karyotype showing a female with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XX, +21

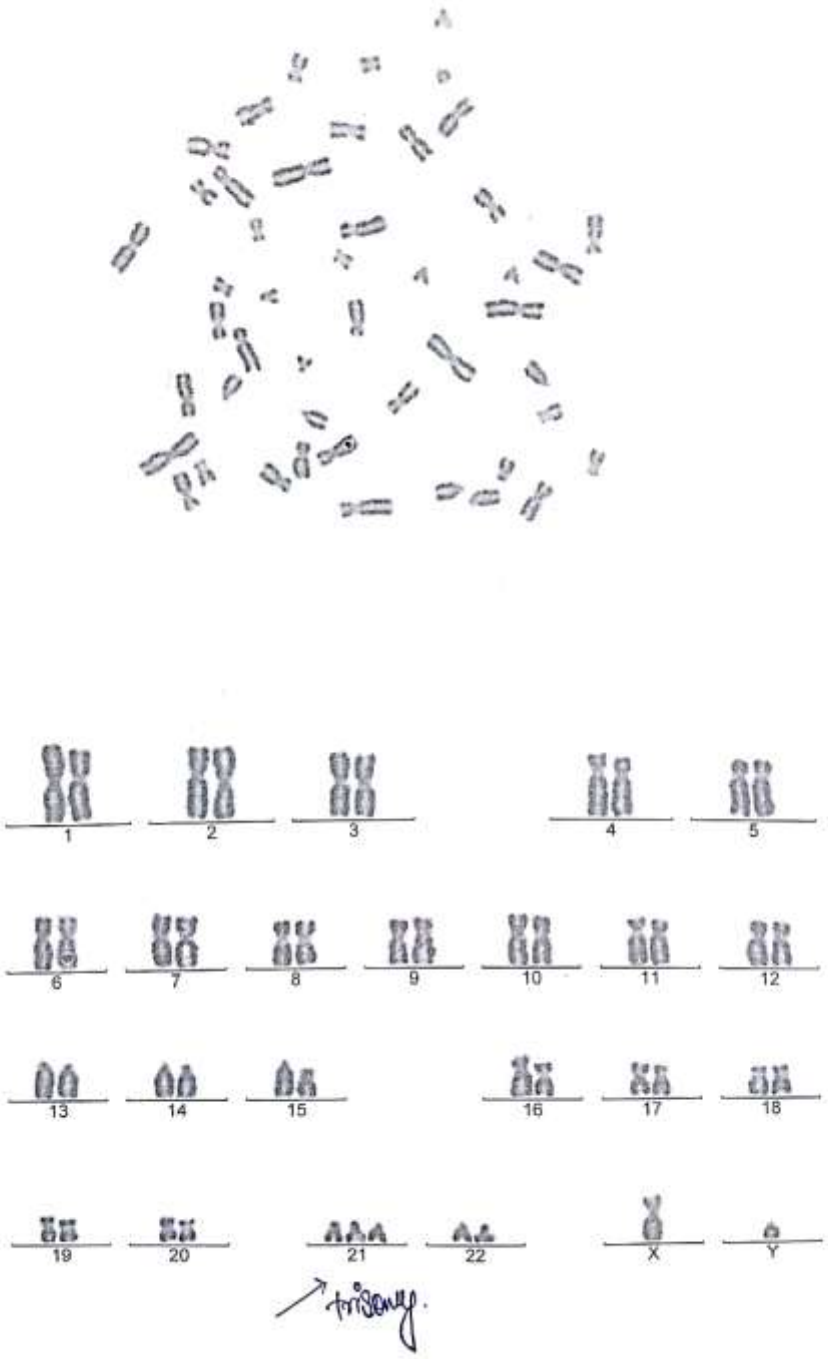


Plate XIII: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21

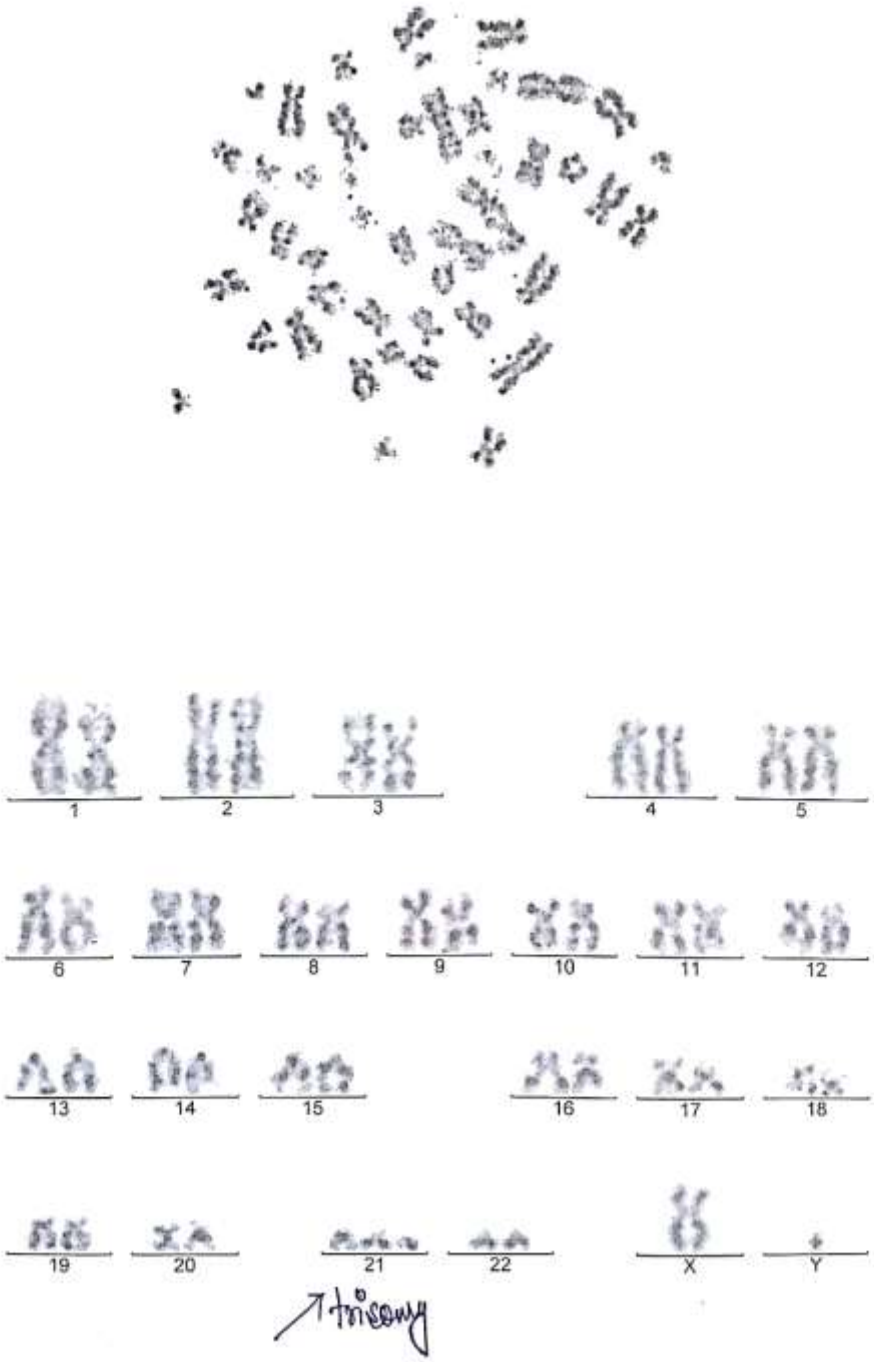


Plate XIV: A karyotype showing a male with trisomy 21 after capturing with Vysis system an arrow indicates trisomy 21. Karyotype result is: 47, XY, +21