"PREVALENCE OF CONGENITAL ANOMALIES IN FETUSES SUBJECTED FOR AUTOPSY – A HOSPITAL BASED CROSS SECTIONAL STUDY"

Submitted by

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Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"PREVALENCE OF CONGENITAL ANOMALIES IN FETUSES SUBJECTED FOR AUTOPSY – HOSPITAL BASED CROSS SECTIONAL STUDY",** is ethical and justifiable.

The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

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LIST OF ABBREVIATIONS

ASD	-	Atrial septal defect
CA	-	Congenital anomaly
CM	-	Congenital malformations
CHD	-	Congenital heart defect
Cms	-	Centimeters
CNS	-	Central nervouS system
CTEV	-	Congenital talipes equino varus
CVS	-	Cardiovascular system
DNA	-	Deoxyribonucleic acid
e.g	-	For example
ECA	-	Extra cardiac anomalies
EDD	-	Expected date of delivery
Gms	-	Grams
IUGR	-	Intrauterine growth retardation
Kg	-	Kilogramn
LBW	-	Low birth weight
MG	-	Meckel Gruber
MRI	-	Magnetic resonance imaging
MTHFR	-	Methylenetetrahydrofolate reductase
N	-	Total number
NFHS	-	National family health survey
NMR	-	Neonatal mortality rate
P	-	Probability
PCKD	-	Polycystic kidney disease

PMR - Perinatal mortality rate

SD - Standard deviation

VSD - Ventricular septal defect

WHO - World Health Organization

Wks - Weeks

Wt - Weight

ABSTRACT

BACKGROUND:

In developed nations, congenital abnormalities are now a significant contributor to prenatal and neonatal mortality, and in developing nations like India, they may soon play an even significant role. Despite antenatal diagnostic methods, foetal autopsies are still extremely important for foetal congenital malformations to be confirmed, identified, and for counselling parents regarding future pregnancies. The direct advantages of autopsy for parents go beyond reducing the likelihood of recurrence. In cases where autopsy cannot reach a final diagnosis, the preservation of foetal samples for potential genetic research in the future offers the possibility of an accurate diagnosis.

OBJECTIVE:

1. To study the prevalence of congenital anomalies in fetuses subjected for perinatal autopsy.
2. To assess the clinical efficiency of autopsy in reaching the final diagnosis.

METHODOLOGY:

160 stillborn autopsies were performed in the Department of Pathology, J. N. Medical College, at KLE's Dr. Prabhakar Kore Hospital, KAHER, Belagavi, Karnataka, from January 2021 to December 2021, where the prevalence of congenital anomalies was described according to the age of the foetus and mother, the predominant system involvement, and a systemwise description of congenital anomalies was done.

Genetic analysis of 20 stillbirth cases was done at the Karnataka Institute for DNA Research (KIDNAR), Dharwad, by analysing the involvement of the MTHFR

gene using DNA isolation, Sanger sequencing, and PCR amplification of the targeted gene.

RESULTS:

Out of a total of 160 cases, congenital anomalies were found in 66 fetuses; hence, the prevalence of congenital anomalies was 41.3% with a female to male ratio of approximately 1.02:1. Three other rare syndromes, like body stalk syndrome, Potter's syndrome, and Dandy Walker syndrome, were also noted. The majority of the foetuses (32.5%) were below 20 weeks of gestation. The most common system involved was the central nervous system, seen in 24 cases (36.4%), followed by miscellaneous in 19 cases (28.7%).

In 20 cases, PCR amplification of Exon-5 of the MTHFR gene revealed positive polymorphisms of the MTHFR gene in 15 cases, confirming the 677 C>T (rs1801133) heterozygous variant.

CONCLUSION:

Foetal autopsy continues to be the gold standard for determining the cause of death despite technological developments. This information is crucial for physicians to manage upcoming pregnancies and provide genetic counselling to parents. The 677C>T single nucleotide polymorphism in the MTHFR gene is associated with poor pregnancy outcomes, and people who carry the T allele are more likely to experience foetal congenital malformations.

KEYWORDS: Perinatal autopsy, Congenital malformation, anomalies, MTHFR gene, Still born, Genetics.

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INTRODUCTION

Perinatal mortality is the most important indicator because it gives information about antenatal and intranatal health, the quality of child healthcare, and the socioeconomic state of the community.[1] Perinatal mortality accounts for about 1.5 percent of all births in developed countries.[2]

Perinatal mortality rate (PMR) is defined as late fetal death (more than or equal to 28 weeks of gestation) and early neonatal death (within seven days of birth) in one year to total number of live births in the same year. It is expressed as rate per 1000 live births[3]. The current Perinatal mortality rate in India is 26 per 1000 births as per the NFHS (National Family Health Survey) .[4]

A stillbirth is defined when a baby is born dead after 24 completed weeks of pregnancy and it accounts for major number of perinatal deaths[5]. Still births are mostly under reported, not studied and seldom considered in an attempt to improvise the adverse pregnancy outcome in developing countries[6].

Nearly 55% of studies registered, definition for still births and the limit between miscarriage and still births varied from 20 weeks of gestation (United Kingdom) to 28 weeks of gestation (India)[6].

Primary obstetric cause of perinatal death include either spontaneous preterm delivery, hypertensive disorders, antepartum hemorrhage, multiple pregnancies, intrapartum asphyxia and other maternal diseases.[7]

A congenital malformation (CM) or birth defect, is defined as any abnormality, either structural or functional, present at birth, which may have been

inherited genetically, acquired during gestation, or inflicted with parturition. The etiology of congenital malformation may be genetic (30-40%) or environmental (5-10%). The cause remains unidentifiable in about 50% of the cases.[8]

Congenital defects are now a significant contributor to perinatal morbidity and mortality in both developing and developed nations.[9] Although most congenital malformations have no recognized cause, there are a few extrinsic risk factors that are well documented and can be avoided. [10] Since the 1960s, a global monitoring programme has been in place to track the emergence of these congenital defects across a range of population categories.[11] Global surveys have revealed that there are significant regional differences in the prevalence of congenital abnormalities at birth.[12] Social, ethnic, ecological, and economic aspects can all be used to account for these discrepancies.[13,14]

To reduce the incidence of congenital anomalies and their prevalence in every population, it is essential that the distribution and prevalence of anomalies should be identified for each and every country, and even for every region.[15]

The mothers usually want to know about the outcome of next pregnancy in future, where the autopsy is quite significant in giving necessary information.[16]

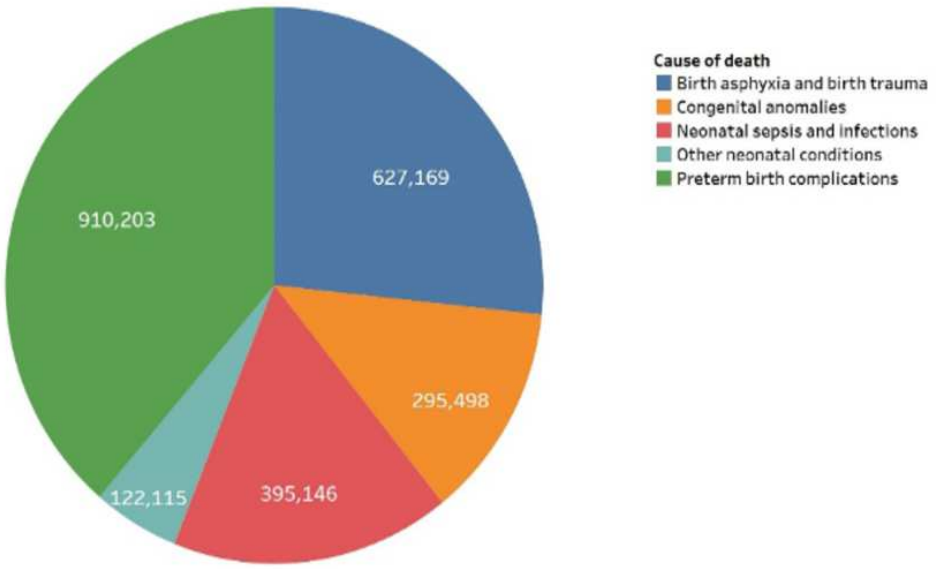


Figure 1: Leading cause of neonatal death globally

The primary goal of the fetal or perinatal autopsy is to identify gestational age of the fetus, document growth and development, detection of any congenital abnormalities, analysis of clinical diagnosis and treatment, and determine the cause of death and rule out possible recurrence risk.[18]

For instance, a fetus with several unexplained deformities may not always require an autopsy if the pregnancy has been terminated and fetal trisomy 18 has been established. A fetal autopsy is definitely warranted if there are any fetal malformations that have been prenatally diagnosed but have not been given a chromosomal cause.[19]

On the incidence of congenital abnormalities in fetal autopsies, there is, however, little information available in the Indian setting. Additionally, no research has been done in this area. In order to ascertain the frequency of congenital abnormalities among fetal autopsies, the current study was designed.

AIMS AND OBJECTIVES

1. To study the prevalence of congenital anomalies in fetuses subjected for perinatal autopsy.

2. To assess the clinical efficiency of autopsy in reaching the final diagnosis.

REVIEW OF LITERATURE

Autopsy is derived from the Greek word autopsia, and means seeing with one's own eyes.

An important factor to determine maternal and child healthcare is by seeing the perinatal mortality rate. In developing countries, the PMR is 3 to 5 times higher as compared to the developed countries.[1-3] The current perinatal mortality rate in India is 26 per 1000 live births[4]. Stillbirths rate in Belagavi is 18 per 1000 births. One half of perinatal deaths are because of stillbirths, with 4 million occurring in worldwide each year.[5] Over 97 percent of these stillbirths occur in underdeveloped nations. In attempts to improve poor pregnancy outcomes in poorer nations, stillbirths have received insufficient research attention, are seldom recorded, and are rarely considered. [1-5]

To unify the criteria of stillbirths, WHO has suggested that the bottom limit for worldwide comparisons be 1000 grammes (corresponding approximately 28 weeks of gestation).[5] The lowest limit for birth weight or gestational age varied greatly. Fetal loss that occurs after 20 weeks of gestation is referred to as a stillbirth in developed nations. However, it was previously decided in wealthy nations (like Sweden) to adopt 28 weeks of gestation as the lower cut-off. The lower cut-off is frequently utilized in underdeveloped nations, where gestational age of 28 weeks or birth weight of 1000 grammes is the standard.[5]

There are differences in the time of stillbirth with respect to delivery. Fresh stillbirths are those that happen during the intrapartum period or right before delivery and do not have macerated skin like those that happen more than 12 to 24 hours

before delivery. Intrapartum stillbirths are a sign of insufficient funding and poor obstetric treatment, respectively.[6]

The prevalence of stillbirths still not completely determined, also the timing and circumstances are unidentified which continue to be associated with stillbirths in developing countries, where one half of all deliveries occur at home.[23] Most of the stillbirth research is hospital based because most of the data on still births are not collected routinely by other countries. Therefore, an understanding of the burden of stillbirth has an imperative inference on health planning system and resources, which are of actual apprehension in very low resource countries.[5]

The most common primary obstetric cause of perinatal deaths include spontaneous preterm delivery (28.7%) and hypertensive disorders (26.3%).[7] Other causes are antepartum haemorrhage, multiple pregnancy, intrapartum asphyxia, fetal abnormality and maternal diseases.[1,7] Preterm birth (30%), sepsis (27%), birth asphyxia (23%) and congenital malformations (6%) are the main cause of deaths in newborn babies worldwide.[24,25]

Fetal abnormalities include stillbirth; perinatal asphyxia; consequences of immaturity; definite infectious or metabolic conditions, and, congenital malformations.[26]

EMBRYOLOGY

Embryonic Stage

The single cell zygote rapidly divides into a ball of cells called a blastula through a process known as cleavage.[27]

The cells organize themselves into two layers, the inner cell mass and the outside layer known as the trophoblast, to form a blastocyst.

The inner cell mass gives rise to an embryo, whereas the trophoblast aids in blastocyst implantation into the endometrium by secreting specific enzymes. It also plays a role in the creation of the placenta, which feeds the growing foetus.

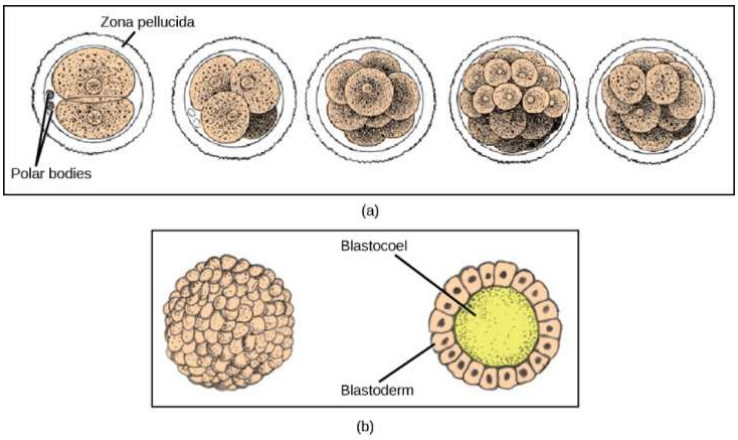


Figure 2: (a) During cleavage, the zygote rapidly divides into multiple cells. (b) The cells rearrange themselves to form a hollow ball called the blastula.

Gastrulation is the term for the process where the cells in the blastula travel spatially and differentiate into three germ layers which are ectoderm, mesoderm, and endoderm.

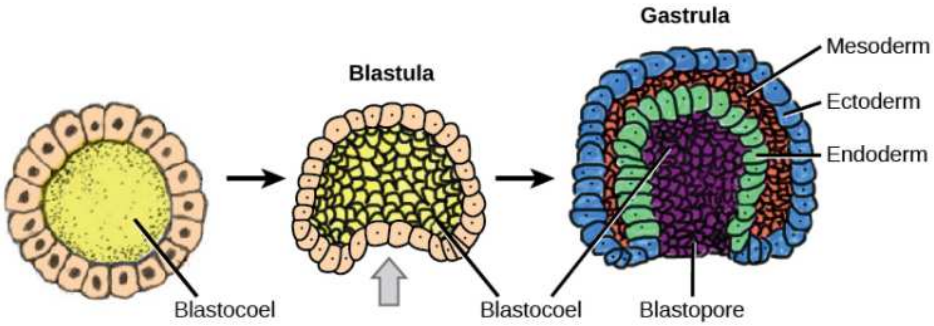


Figure 3: Gastrulation – Formation of the three germ layers

In particular:

- The gastrointestinal and respiratory systems, as well as the thymus, parathyroid, bladder, and urethra, are all derived from the endoderm.
- The neurological system, a component of the sensory organs, and the skin and its appendages are all developed by the ectoderm.
- The mesoderm is responsible for the formation of many internal organs, the lymphatic system, bone, cartilage, and muscles. Among them are the adrenal cortex, kidney, spleen, and ureters.

NEURULATION

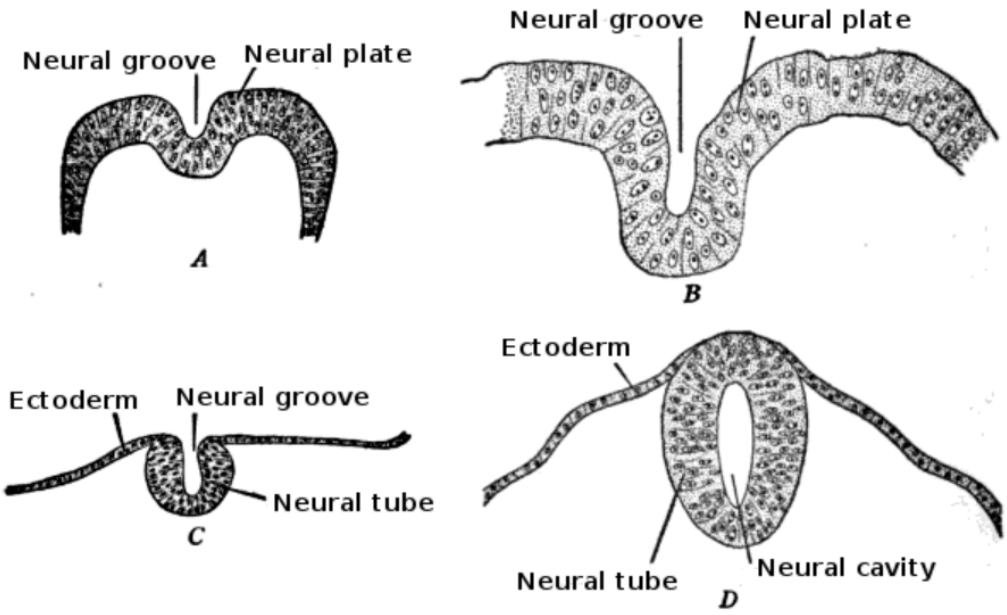


Figure 4: Process of formation of neural tube

Neurulation is a process of neural tube formation by the ectoderm layer, which begins between the 3rd and 4th weeks of gestation and is marked by the development of a notochord-like bulge in the middle of the mesoderm. The neural plate, a thickening above the endoderm known as the notochord, is created by the secretion of

stimulating and differentiating substances by the notochord. Later, the neural plate folds outward, and the fusing of the two ends results in the creation of the neural crest.[28]

The neural crest is detached from the epidermis by the closure of the neural tube, and the neural crest cells oversee forming the majority of the peripheral nervous system's components. Myelomeningocele, anencephaly, and encephalocele are examples of defects in neural folding and neuropore closure.[29]

ORGANOGENESIS

Most of the organ development occurs between the third and eighth weeks after fertilization.[30]

DEVELOPMENT

Cardiovascular System[30]

The cardiovascular system is the first organ system to form during organogenesis. In contrast to week six, which sees cardiac outflow separation and the descent of the heart (and lungs) into the thorax, week four of development sees the establishment of the heart's four chambers. The ascending aorta and pulmonary artery are separated from the truncus arteriosus through spiraling of the aorticopulmonary septum. Anatomically, the aorta and pulmonary artery seem to encircle one another above the heart. Embryologic swirling is the cause of its appearance. The spiral or conotruncal septum is another name for the aorticopulmonary septum.[30]

Lung Development

From the embryonic stage through the foetal stage and up until delivery, the lungs continue to grow. The formation of the right and left lung buds from the respiratory diverticulum, the first outpouching, marks the beginning of lung expansion in the early embryo. The respiratory tree is formed by the buds as they grow and branch. Weeks five through seven are when the visceral and parietal pleura begin to show. Mesoderm is the source of both varieties of pleura. The interior chest wall is covered by the parietal pleura, whereas the visceral pleura covers the growing bronchial tree. The diaphragm, which divides the pleural and peritoneal body cavities, forms and fuses with pleuroperitoneal membranes. By around week seven, these membranes have finished sealing the pleuroperitoneal canal.[30]

Central Nervous System

The neural tube closes around week four and is the only derivative of brain and spinal cord. The CNS develops its vesicles, which are embryological precursors of various brain regions, throughout weeks five through eight of pregnancy. Vesicles give rise to the forebrain, midbrain, and hindbrain.

These are called as prosencephalon, mesencephalon, and rhombencephalon. The diencephalon and telencephalon later emerge from the prosencephalon. The telencephalon develops to surround the diencephalon, midbrain, and hindbrain, while the thalami, hypothalamus, optic cups, and neurohypophysis are all born from the diencephalon. The mesencephalon creates the superior and inferior colliculi, the aqueduct of Sylvius, and the tegmentum. The fourth ventricle and the metencephalon, a structure that later gives rise to the pons and cerebellum, both emerge from the rhombencephalon.[30]

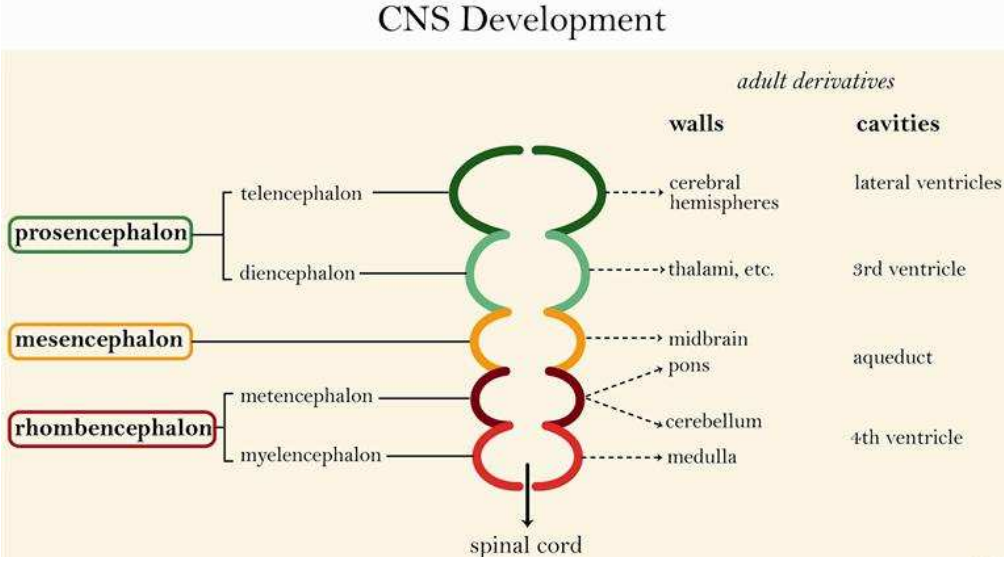


Figure 5: Process of CNS development

Gastrointestinal System

Weeks six through eight are critical for the development of the gastrointestinal system. The midgut undergoes physiologic herniation through the umbilicus around week six. This physiologic process happens because the size of the abdominal cavity is too small to accommodate the enlarging gastrointestinal tract. Herniation provides ample space for the rapidly enlarging midgut. After herniation, the midgut undergoes three rotational events totaling 270 degrees of rotation. The first rotation consists of 90 degrees in a counterclockwise direction around the superior mesenteric artery. This helps establish the appropriate arrangement and placement of the bowel; the ileum is brought to the right side of the body. The second rotation occurs during 10 weeks of gestation and consists of 180 degrees in a counterclockwise direction. The midgut returns to the body cavity at the end of 10 weeks. Finally, the third rotation of 180 degrees in a counterclockwise direction places the cecum on the right side.

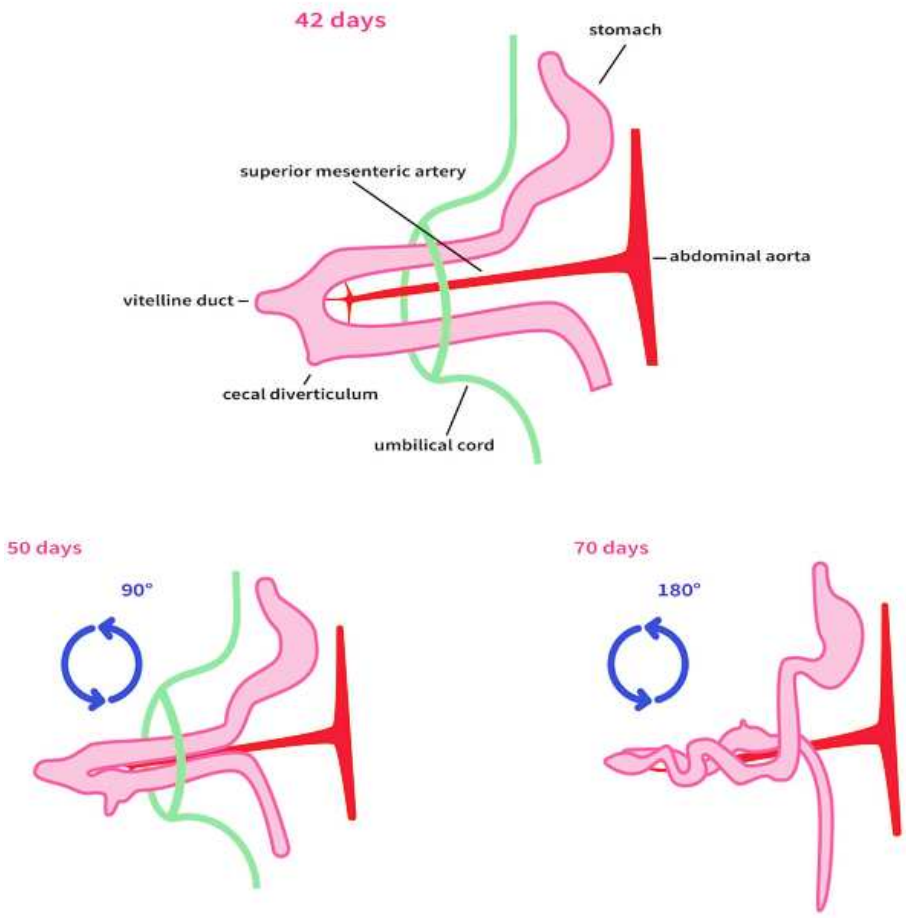


Figure 6: Stages of embryonic rotation of the gut

Other Organs

Many other organs develop during weeks six through eight, including the pituitary gland, thymus, and adrenal cortex. At week seven, the embryo assumes a characteristic C-shape. At week seven, the ocular retina also begins to develop. The upper and lower limbs continue to grow. Also, facial structures such as the nostrils, eyelids, outer ears, lip, and palate develop, and at week seven, the head and face contours begin to emerge.

Congenital Malformations

Diseases that are mostly predetermined before or during birth and that are theoretically detectable in infancy are referred to as congenital disorders. Congenital malformations, according to a WHO statement from 1972, should only refer to structural birth defects, while congenital anomaly should be used to refer to all biochemical, structural, and functional birth defects.[31]

A birth abnormality affects 2.5% of babies in India, according to population and hospital surveys, and this number rises to 4% of children if they are tracked up to age five. In India, congenital malformations are already the third most common cause of perinatal mortality.[32]

An aberration of structure, function, or body metabolism that is present from birth (even if it is not discovered until later in life) and causes physical or mental handicap, or is fatal, is referred to as a congenital anomaly.[33]

When there appears to be a genetic component but no obvious mendelian pattern of inheritance, multifactorial inheritance is considered.

Classification of congenital anomalies [(34-37)]

Congenital abnormalities are now categorized in a variety of ways as our knowledge of pathophysiology and causation has grown.

A *malformation* is a non-progressive, congenital morphologic anomaly of a single organ or body part due to an alteration of the primary developmental program.

Deformation is an altered shape or position of a body part due to aberrant mechanical force(s) that distorts an otherwise normal structure.

A morphologic abnormality known as *disruption* results from extrinsic interference with a normally occurring process.

Dysplasia: improper tissue organization

A *syndrome* is defined as "a recognized pattern of anomalies that are known or believed to be causally related".[34]

Sequence: "series of aberrations resulting from a known malformation"[35]

Complexity is defined as "those groups of complex illnesses with overlapping characteristics that are difficult to divide into individual syndromes," for example, the facio-auriculo-vertebral spectrum and hypoglossia-hypodactylia.[36]

Association: A pattern of anomalies, at least two of which are morphologic, that occur together more often than would be expected by chance, and where a causal relationship has not been identified.[37]

FETAL AUTOPSY

Autopsies have been used in medicine since the 15th century and have considerably contributed to clinical knowledge.[38]

They play a particularly important role in the counselling of families following the loss of a baby since they may aid in the grieving process and increase parental comprehension. It is also possible to detect genetic abnormalities or obstetric factors that are relevant to future pregnancies. The primary goals of autopsy examination are the identification of the cause(s) of death, the elucidation of the pathogenic mechanism, and the quality control of the clinical mechanism.[38]

The autopsy can offer helpful explanations, and it enables medical professionals to provide the family with more accurate genetic counselling and to prepare for the management of future pregnancies. When there is a foetal abnormality or when there is no definitive clinical diagnosis, autopsy results are more likely to be helpful.[39]

Demonstrated benefits of a fetal or perinatal autopsy

By comparing the clinical and autopsy diagnoses in stillbirths, neonatal deaths, and therapeutic terminations, Gordijn et al.[40] assessed the effectiveness of perinatal autopsies. In 22% to 76% of cases, the autopsy showed a change in diagnosis or added new information. If clinical findings are also taken into account, perinatal autopsies may have been useful in 100% of cases.

In 91 autopsies conducted after antenatal diagnosis of foetal abnormalities, Phadke and Gupta[41] later found comparable indications of performance: foetal autopsy produced a definitive diagnosis in 79.1% of the cases and supported the sonographic findings in 97.8% of the instances. In 33% of the cases, new information assisted in reframing the diagnosis.

A series of 1012 successive terminations for foetal abnormalities was published by Dickinson et al.[42] In 809 cases, an autopsy was conducted in 63.5% of cases, autopsy provided no new information beyond the prenatal diagnosis (357 of 562). Autopsy gave considerable information in 15.1% (85 cases) and supplied major diagnostic information in 1.1% (6 cases). In 16% of cases, autopsy helped to identify or explain some prenatal abnormalities.

In 27% of the instances, previous research claimed that the recurrence risk had been improved overall. As a result, in about 30% of perinatal postmortem examinations, new evidence from the autopsy modifies the underlying diagnosis or the information given to the parents during counselling.[43]

When the autopsy is conducted as soon as possible, following the foetal death, the findings are more likely to provide additional information.[39] Clinicians can, with certainty inform parents the value of an autopsy in determining the reason of death and in guiding them through any subsequent pregnancies.[44]

Obtaining consent for autopsy

The parents must have clear information in order for them to give informed consent for a full or limited autopsy. When pregnancy termination is first being discussed, the subject of a postmortem autopsy may come up.

The medical staff should provide the family a thorough and timely explanation of the autopsy in a calm setting, giving them enough time to ask questions.[22]

In addition, written material on the perinatal autopsy must be made available to parents. As important as being proficient in collecting the specimens and carrying out the autopsy, the capacity to be empathetic while explaining the justification for the postmortem examination and alternatives to parents is equally important.[44]

It is important to let the parents know that their child will always be treated with respect and decency. According to the recommendations made by the European Parliament and Council, postmortem consent forms should have a section that specifically addresses the question of organ retention.[22]

Up to 60% of parents have reportedly agreed to organ retention. Depending on the jurisdiction, different laws may apply to fetal autopsies. Alternatives to autopsy, must be given if the parents refuse to give their permission for a full autopsy.[22]

Alternatives when a family declines an autopsy

The controversies surrounding the issue of organ retention are likely to have had an impact. The year after the implementation of a guideline for investigating stillbirths in Alberta there was an increase from 54% to 74.5% in fetal autopsies and a decrease to 48%.[39] A similar decrease has been reported in other countries. Khong and Tanner reported a 58% acceptance for fetal autopsy in a group of 305 women following pregnancy terminations.[45]

Autopsy may be declined due to -

a) the parents feel the baby has already suffered enough,
b) the parents thinks that prenatal investigations were sufficient,
c) health care professionals failed to provide adequate explanation of autopsy.

Cultural and religious considerations pertaining to fetal and perinatal autopsies are reviewed in the literature. The treating team should respect the parent's decision when parents are reluctant to consent to a full autopsy, they may agree to a limited autopsy, including examination of specific body cavities, or full body imaging techniques, which will allow specific questions or concerns to be addressed and which may be more acceptable to some families.[22]

MRI may be offered to parents who decline an autopsy investigation, although the limited availability of MRI and the need for prioritization are concerns in most

countries. Clinicians should explain to the parents that a full autopsy remains the gold standard because, the MRI does not provide important information and thus a lot may be missed. Many limitations of using perinatal and post-mortem MRI are cited in the literature: high cost, limited availability, lack of experience, need for specialist equipment, lower resolution, lack of detection of changes at the histological level, and uncertain value when there is an advanced degree of maceration or autolysis.[46]

A 2008 overview underlined the non-invasive nature of the MRI examination and the detection of pathologies and malformations of the central nervous system.There was a complete agreement in 60% of cases between MRI and autopsy findings. The autopsy is essential in finding the cause of death in 36% of the cases. If MRI had been the only investigation, essential information would have been lost in 17 of 24 cases (71%).[46]

Another smaller study (n = 26) comparing post-mortem MRI and autopsy for all malformations demonstrated an 80% detection rate for major malformations and 10% detection rate for minor malformations[47]

A recent meta-analysis comparing the performance of MRI with that of conventional autopsies demonstrated a 69% sensitivity and 95% specificity in determining the final cause of death or most clinically significant abnormality in 146 fetuses. Well-designed large prospective studies are needed to evaluate the accuracy of post-mortem MRI.[48]

Therefore, the integrated result obtained from the traditional autopsy remains crucial in determining the cause of the malformation for fetal or perinatal death.

All fetuses with known congenital malformations should have at least cytogenetic analyses performed. This analysis can be done on cord blood, fetal tissues, or the placenta. Fetal tissues and placenta are a good source of fetal DNA that can be banked for further studies as indicated.[22]

AUTOPSY TECHNIQUES[49]

Four principal autopsy techniques can be distinguished:

TECHNIQUE OF R.VIRCHOW-

Organs are removed one by one. This method has been used most widely, often with some modifications. Originally, the first step was to expose the cranial cavity and, from the back, the spinal cord, followed by the thoracic, cervical, and abdominal organs, in that order.

TECHNIQUE OF C. ROKITANSKY

This technique is characterized by in situ dissection, in part combined with the removal of organ blocks. Only second-hand descriptions are available. The term "Rokitansky's technique" is used erroneously by many pathologists to designate the removal techniques by Ghon and Letulle, as described in the next paragraphs.

TECHNIQUEOF A. GHON

Thoracic and cervical organs, abdominal organs, and the urogenital system are removed as organs blocks ("en bloc" removal). Modifications of this technique are now widely used.

TECHNIQUE OF M. LETULLE

Thoracic, cervical, abdominal, and pelvic organs are removed as one organ block ("en masse" removal) and subsequently dissected into organ blocks . This technique requires more experience than the other methods but has the great advantage that the body can be made available to the undertaker in less that 30 min without having to rush the dissection.

Fetal measurements[50]

Table 1: Weight and measurements of fetuses of 8 – 26 weeks gestation (Mean Values)

Gestation (weeks)	Weight (g)	Crown-heel length (cm)	Crown-rump length (cm)	Foot Length (cm)
8	10	2		
9	11	3		
10	14	4		
11	18	6	4	0.9
12	25	7	6	1.1
13	27	9	7	1.4
14	38	10	8	1.7
15	53	13	9	2.1
16	73	14	10	2.2
17	122	17	12	2.4
18	161	19	13	2.6
19	188	20	14	2.9
20	227	21	15	3.2
21	303	24	16	3.4
22	384	26	18	3.8
24	389	27	19	4.1
26	394	28	20	4.5

The Abortion Act 1967 allows termination of pregnancy if there is substantial risk that if the child was born it would suffer from such physical or mental

abnormalities . During the past few years , new screening and diagnostic tests have been formulated that allow earlier and more detailed diagnosis of fetal anomalies.[51,52]

After termination of pregnancy most parents generally wants to know if the prenatal prediction was right and what are the implications for future pregnancies. This is particularly true when the prenatal diagnosis is based on ultrasound scan findings only. Such information may be obtained from the autopsy examination.

In a study by Boyd, Tondi and Hicks, on autopsy after termination of pregnancy for fetal anomaly,[51] showed that out of 57,258 deliveries, 309 (0.5%) were terminated because of prenatally diagnosed abnormality. There were 129/29 ,86 (0.4%) terminations for fetal anomaly carried out in 1991-5 and 180/28 ,172 (0.6%) in 1996-2000. The percentage of fetuses that underwent autopsy fell from 84% to 67%. Autopsy was performed in 132 cases identified by the ultrasound scan, with no evidence for abnormal karyotype. In 95 (72%) the autopsy confirmed the suspected diagnosis and didn't add important further information, two cases were not classified, and in 35 (27%) the autopsy added information that led to a refinement of the risk of recurrence.

When a prenatal diagnosis was based on the results of a scan only, the addition of information from an autopsy by a specialist paediatric pathologist provided important information that changed the estimated risk of recurrence in 27% of cases and in 8% this was to a higher (one in four) risk.[51]

Chromosome anomalies accounted for 141 (46%) of the 309 cases. Neural tube defects and other central nervous system defects were the most common structural defects, accounting for a further 66 (21%) cases.

Quantifying the value of autopsy is not easy. For example, in many cases of lethal skeletal dysplasia a diagnosis can be attempted prenatally, but confirmation is needed from autopsy and x-ray studies and these may change the suspected risk from low (for example, thanatophoric dysplasia) to high (for example, Jeune's syndrome) recurrence. Similarly renal cystic disease may be difficult to define on a scan because of a lack of amniotic fluid, and the differentiation between infantile polycystic kidney disease (recurrence risk 25%) and cystic renal dysplasia (recurrence risk 3%) may require histological examination.[53]

In a study to identify trends in neonatal autopsy rates at a tertiary care hospital during the last decade, of 487 neonatal deaths, autopsies were performed in 296 (61%) cases. The autopsy rate declined significantly during 10 years from 71.2% (1984-1988) to 47.7% (1989-1993). New diagnoses were made at autopsy in 44% of cases. Major discordances were identified in 35 infants (12%) and minor discordances in 95 (32%). Autopsies were more likely to reveal new diagnosis in infants born at 28 to 36 weeks gestation and in those whose mothers had no prenatal care. Major findings at autopsy were more ore likely in infants whose mothers had no prenatal care and in infants who died within 6 hours of birth. This study revealed a significant decline in neonatal autopsies during a 10-year period. This study also demonstrates that neonatal autopsy continues to provide clinically significant data and remains a valuable tool in perinatal medicine.[54]

Michele Harrison and Hourihane[55] enumerated the factors contributing to the decline in autopsy rates. They include the following.

1. The findings may not be considered as relevant by the clinician.

2. The procedure may be poorly performed by a trainee pathologist who is insufficiently supervised or it may be done by a rushed pathologist, anxious to deal with other duties.

3. The results may not be sent back to clinician for several months after autopsy has been performed.

4. There may not be direct contact with the clinician or with the family of the deceased.

Wigglesworth JS[56] reported that referral of perinatal deaths to a regional center for postmortem examination is becoming more common ,partly because the opinion obtained is thought to be of more value. He says that the public interest will be furthered in two ways; firstly, there would be an independent reassurance for bereaved parents and the public that such death had occurred despite of the medical intervention and secondly, the higher perinatal autopsy rate would help to maintain clinical standards, monitor the benefits and harm done by modern technology and provide on often needed factual basis of genetic counselling.

According to Porter HJ et al[57] a retrospective study of 150 still births and 150 neonatal deaths was carried out between 1981 and 1985, the necropsy findings were compared with clinical diagnosis. In all cases the autopsy comprised of macroscopic findings and histological examination of all organs, with microbiology, radiology and cytogenetic.

Clinically important differences between clinical and pathological diagnosis in 54 of 150 cases (36%) were noted in the cases of still births. Of the neonatal deaths, examination showed clinically important information that had not been recognized

during life in 66 cases (44%). Despite dramatic changes in medical techniques of investigation and treatment, 10% of autopsies showed major pathological abnormalities which if recognized before death would have led to change in management and longer survival of the baby.[57]

Sharma AK[58] reported that the planning of genetic counselling and consideration of possibility of prenatal diagnosis in a future pregnancy should begin in doctor's mind as soon as an abnormal baby is delivered, many doctors imagine that the suggestions of an autopsy will distress parents very greatly, but this is not usually the case, if the reason for the autopsy are properly explained then there's a high chance that parents will consider the autopsy procedure.

A total of 2063 live births were examined between July 1994 and June 1995 in a study by Kaushik et al.[59] to examine the association between the neonatal mortality rate and birth weight and gestational age. The NMR, or neonatal mortality rate, was 35.4 per 1,000 live births. Low birth weight and preterm infants had case fatality rates of 10.1% and 18.1%, respectively. However, low birth weight infants made up 27.8% of the population.

In order to determine the overall frequency of congenital malformations in a city hospital in the first three days of life, Patel ZM et al.[60] conducted birth defects surveillance research. Pediatricians and geneticists assessed and made diagnoses on 17,653 consecutive new-borns in a maternity facility. On a pre-made proforma, pertinent data was recorded and examined 294 (1.6%) of the 17,653 babies which had serious abnormalities, while 1400 (7.92%) had minor ones, 328 (1.8%) of the 17,653 births were stillbirths.

Table 2: Correlation of Gestational Age with Malformations

Gestational age (weeks)	**No. of malformed babies**	**No. of normal babies**	**Percentage**
<28	*6*	37	16.20
28 to 31	11	133	8.20
32 to 36	37	644	5.76
Full term	239	16808	1.48
>42	1	31	3.2
TOTAL	**294**	**17.653**	

Table 3: Correlation of birth weight with malformations

Birth Weight (Kg)	**Malformed babies**	**Normal Babies**	**Percentage**
<1	12	167	7.18
1-1.49	20	440	4.4
1.5 – 1.99	29	1131	2.56
2.0 – 2.49	84	4452	1.88
1.5 – 2.49	101	7701	1.31
3.00 – 3.49	37	3016	1.22
3.5 – 3.99	10	*718*	1.39
>4	1	28	3.57
Total	**294**	**17,653**	**1.67**

Nybo Andersen et al[60,61] reported that fetal loss increases with increase in age of the mother, irrespective of their reproduction profile. Women's older than 30 years have significantly higher risk of spontaneous abortion, ectopic pregnancy, stillbirth and congenital anomalies . The risk of spontaneous abortion of fetus in women at age 20 –24 is 8% whereas the risk has increased to 71% in women above 45 years of age. Therefore, maternal age is independently quite a strong factor responsible for fetal loss and congenital anomalies.

Banerjee CK et al[62] reported in his study regarding the incidence of cause of death in fetuses. The commonest cause were the prematurity and low birth weight. Two most commonly affected systems for congenital anomalies were the cardiovascular and the genitourinary.

Machin GA[63] reported on perinatal mortality survey conducted of 726 infants in southeast London in which out of 185 antepartum death (25%), intra partum deaths (22%) were 161 and early neonatal deaths were 380 (53%). Malformation were the largest group, followed by antepartum and intrapartum hypoxic deaths. Male babies predominated among hypoxic and traumatic deaths and among premature neonates. The majority of perinatal deaths from malformations occurred in neonates but 11% of antenatal death had lethal malformations. Intrapartum hypoxia was the most common cause of intrapartum death but was also responsible for 15% of neonatal deaths. Intracranial traumatic lesions accounted for only 4% of neonatal deaths. Prematurity caused 1/3 of all early neonatal deaths while a further 1/4 neonates were malformed. Intra ventricular hemorrhage was found in 20% of neonates and in 11% of all perinatal deaths. Eight infants had died of extra pulmonary infections.

In an autopsy study of 103 perinatal deaths by Chowdary P et al,[64] it was found that asphyxia, congenital anomalies and infection was the primary causes of death. 31 deaths were reported due to asphyxia, congenital anomalies were reported in 21, two babies had infection and other two baby were extremely premature (birth weight less than 1000gms). Other causes were as follows ; 7 cases of pulmonary hemorrhage, 4 cases of amniotic fluid aspiration, 2 cases of Hyaline membrane disease , 1 case of hydrops fetalis and 1 case of adrenal hemorrhage . The 4 babies were macerated and no cause of death was identifiable in those 10 cases. They noted that approximately 70% of deaths were due to reduced antenatal checkups by mothers and around 50% the mothers were primigravida and 65% of babies were born before 37 weeks of gestation period.

In an autopsy study of 100 cases, Joshi VV[65] described the subsequent incidence of principal causes of death. The author also noted that the respiratory distress syndrome was the leading cause of death among low birth weight (less than 2,500 grams) babies and the next common cause were the extrinsic perinatal hypoxia and congenital malformation. In group of full birth weight (above 2,500 grams) the congenital anomalies and extrinsic perinatal hypoxia occupies the first and second place.

The most common congenital anomalies were those of cardiovascular anomalies and second prevalent cause were the multiple congenital anomalies.

In a study on congenital malformations at birth by Swain et al,[66] 3932 consecutive newborns were examined at birth for the presence of congenital malformations. Major organ system involved was the central nervous system (39.5%) followed by the musculoskeletal system (14.5%). Of 48 cases, 39 (81.2%) had

involvement of single system and 9 (18.8%) had involvement of more than one system.

Association of more than one organ system was observed in 18.8% case. The the babies' born to mothers of gravida four or more had strikingly greater incidence of malformations as compared to mothers of lower gravida.

Table 4 : Type of congenital malformations observed

SYSTEM	TYPE	MALFORMATIONS	No.	%
Central nervous			19	39.5
System	Major	Anencephaly	6	12.5
		Hydrocephalus	8	16.6
		Meningomyelocele	3	6.2
		Microcephaly	1	2.0
		Encephalocele	1	2.0
Musculoskeletal			7	14.5
	Major	Osteogenesis imperfecta	1	
	Minor	Talipes	5	10.0
		Congenital dislocation of hip	1	2.0
Gastrointestinal			5	10.4
	Major	Tracheo- esophageal fistula	1	2.0
		Exomphalos	3	6.2
		Diaphragmatic hernia	1	2.0

According to the study of Stubblefield PG et al[67] at Boston Hospital for Women, they observed, perinatal deaths were the commonest in preterm babies. Out of 442 perinatal deaths, 64 were either due to term or post term babies. They also found that the commonest cause of death in the preterm infants was respiratory distress syndrome while for term babies it was extrinsic perinatal hypoxia which was about 56.1%. Fatal congenital anomaly was noted in 26.3% of cases , infection in 5.2%, intra uterine growth retardation in 3.5%, trauma during birth in 1.8% of cases and unexplained in 5.3%. Extrinsic perinatal hypoxia was observed in 71.4% cases of post term infants.

According to the study made by Sharma M et al[68] at Enana Hospital, Jaipur, hyaline membrane disease was the important cause of neonatal deaths accounting for 36.1% (183 out of 507). The other causes were prematurity 25.4%, neonatal septicaemia 17.4%, birth anoxia 10.4%, intra cranial injury 4%, aspiration pneumonia 4.1%, hemorrhagic disease of newborn 0.98%, congenital anomalies 0.59% and bronchopneumonia 0.39%. They observed that majority of death was associated with increased maternal age, beyond 30 years and less than 20 years.

Nakumara Y et al[69] in their autopsy study of 1000 cases at Kurume University School of Medicine noted that, neonates infection are the major cause of perinatal death present in 35.8%. Hyaline membrane disease was the next common cause seen on 26.1% cases followed by congenital malformation 18.4%, anoxia 10.3%, immaturity 3% and unaccounted 3.4%. In case of still birth, anoxia was seen in 33.5%, malformations 29.0%, maternal cause 8.5%, other cause 6.5% and unaccounted 18.5%.

In a clinical study at the Mines Hospital, Bihar, by Gupta PK et al[70] reported that the most common cause of perinatal death accounting for 60.5% cases was asphyxia, with or without association of maternal complications like pregnancy induced hypertension or antepartum hemorrhage, followed by infections 10.9%, 7.3% had respiratory distress syndrome , congenital malformations in 4.8%.

According to Kumari S et al[71] postdated pregnancy is an immediate risk factor for perinatal mortality. The percentage of fetal distress and birth asphyxia was significantly increased in post-dated groups as compared to the control groups. The perinatal mortality was increased by 1.5 times than those of the control ,in group I (within 7 day EDD), 3 times higher in group II (8-14 days after EDD) and 4 times greater in group III (more than 14 days after EDD).

According to Barson AJ et al[72], the babies with birth weight between 1 to 1.5 kg had reduced perinatal mortality and surprisingly the cases of asphyxia were dropped as a cause of perinatal death because of earlier diagnosis of fetal distress. There was also reduction in deaths because of congenital malformations and macerated still births. They attributed this to the improved perinatal care with advanced intensive neonatal care units, prenatal and antenatal screening techniques.

In a study of 597 perinatal deaths, in one year at SAT hospital, Trivandrum, Bai NS et al[73] noted early neonatal death , during the first 24 hours of life accounted for 31 % of cases and 9% of the babies were those which weighed less than 1000 grams; a preponderance of the male babies in both still birth and neonatal death and 10.05% cases of perinatal deaths was constituted by multiple births. Birth asphyxia was the commonest cause of death(31.28%) followed by prematurity (15.6%), lethal congenital malformations (8.4%) and infections (7.2%).

Singh M et al,[74] in their study at All India Institute of Medical Sciences noted the following causes of death in the perinatal mortality of 411 babies which are as follows: 28.7% perinatal hypoxia, 18.9% immaturity, 14.6% congenital malformations, 5.8% hyaline membrane disease, 5.6% infections, 4.6% other specific causes and unknown in 21.7%.

In a clinical study, Kameshwaran C et al[75] noted that still births accounted for nearly 62% in a total of 211 perinatal deaths. They described that asphyxia was the most common cause of still births, followed by the congenital anomalies, Rh incompatibility and infections.

To determine the prevalence of omphalocele, a retrospective, case-series study carried out by Ortegón-López AJ et al.[76] between 2007 and 2019 found that the prevalence of the defect was 1 case per 1000 newborns in the period studied. The average age of the mothers was 26.7 (15 to 41) years, and the weeks of pregnancy at birth were 35.6 (25 to 41.1). A giant omphalocele was found in 39 patients. 37 cases were also related to other congenital malformations associated with omphalocele.

In comparison to the general population, newborns with omphalocele had a CHD incidence that was 30 times higher. Additionally, compared to cases of omphalocele alone, these infants with omphalocele linked with CHD had a greater frequency of numerous congenital abnormalities with or without any syndrome.[77]

According to Tibrewala NS et al[78] about 2% of infants are born with abnormalities that fatal or capable of causing death if left untreated. Out of 24 fatalities that occurred during their study, 4 were due to anencephaly, 3 due to spina bifida aperta, 4 due to tracheo-esophageal fistula, two cases were of intestinal atresia,

one case of exomphalos, and down's syndrome, ventricular septal defect, diaphragmatic hernia, trisomy 17-18, and achondroplasia.

Greenwood RD, et al[79] studied the presence of various extra-cardiac anomalies that were associated with congenital heart disease. Their study revealed that of the 1566 cases of CHD, 385(25.2%) had additional significant extra cardiac anomalies. 60% of those with extra cardiac anomaly had more than one system involvement in addition to cardiovascular system. The frequency of ECA in symptomatic cardiac infants according to affected system were as follows:

Table 5: Frequency of ECA in symptomatic cardiac infants

SYSTEM	No	PERCENTAGE
Musculoskeletal	137	8.8
Specific syndromes	132	8.5
CNS	107	6.9
Renal	83	5.3
Gastrointestinal	65	4.2
Respiratory	58	3.8
Endocrine	21	1.3
Immune	10	<1
Reproductive	1	<1
Others	45	2.9

Greenwood RD,[80] reviewed cases of congenital diaphragmatic hernia in 48 babies. The authors noted that in babies with congenital diaphragmatic hernia, the extra cardiac anomalies were uncommon. On contrary to infants who had cardiac abnormalities, they found 46% had presented with an ECA like omphalocele, down's syndrome, syndactyly, hypospadias.

Saxena HMR et al,[81] noted in their study of an autopsy in perinatal deaths at Safdarjang Hospital, New Delhi, that about 18.1% of the perinatal deaths had congenital deformities involving one or more system. CNS anomalies were the highest contributing to 35.4% and 28.3% showed malformations of gastro-intestinal tract, 19.3 % involved the CVS.

In a study of congenital anomalies in perinatal mortality done by Chopra J et al[82] on 6375 births over a period of 8 months. Out of 457 perinatal deaths detailed autopsy was done on 220 cases with an autopsy rate of 46%. 24 cases were already reported of congenital malformations on clinical examination. During detailed autopsy, additional 26 more cases were detected of congenital anomalies which clearly ascertained the role of autopsy in diagnosing the cause of death in those undiagnosed cases clinically.

In a 3 year study of Anuja B, Krutika B[83] reported that congenital malformations were seen in women with age group (55.7%) between 21 years and 30 years, and more commonly (2.57%) in the multiparae in comparison with the primiparae (0.42%). It was seen that majority of congenital anomalies were associated with low birth weight (LBW), prematurity, multiparity, and consanguinity.

Vandana M[84] studied a total of 217 fetal autopsy cases, out of which 51 was anomalous. Most common cause of death found in autopsy examination is meconium aspiration in male fetus and placental insufficiency in female fetus. And mean age of gestation is 29 week and 30 weeks respectively.

In their study of congenital abnormalities in live-born newborns, Kulshrestha R et al[85] concluded that the incidence of deformity is 3.41%. Major birth abnormalities were the cause of nearly 15% of newborn fatalities and 26% of deaths in the third trimester. The gastrointestinal tract, the genitourinary system, the central nervous system, the cardiovascular system, and the organs of the special senses were the most often affected systems.

Congenital Anomalies and Genetics

The causes of these anomalies are grouped into genetic (50%) which includes single gene disorders (7%), chromosomal disorders (8%), multifactorial (25%); and environmental factors (10%). The causes are not known for nearly 50% of the congenital anomalies i.e., idiopathic.

Genetic disorders are classified into 3 main groups:[87]

A) Single-gene,

b) Chromosomal,

c) Multifactorial disorders.

Single gene /Mendelian disorders are due to error in DNA sequence of a gene and it can be autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XR), X-linked dominant or Y-linked (holandric) disorders. Chromosomal disorders result due to the chromosomal aberrations which includes numerical and

structural damage. Techniques like, molecular and cytogenetics have been applied to identify these genetic mutations causing various diseases.[87]

Oogenesis begins in the female fetus at 12 weeks, but it is stopped in a stage of meiosis I (when the homologous chromosomes have replicated and paired as bivalents or tetrads) at about 20 weeks.[88] At puberty usually only one oocyte is released per month; a primary oocyte completes meiosis I and produces one secondary oocyte and one polar body.

Chromosomal aberrations including numerical (due to errors at chromosome pairing and crossing-over) and structural damages lead to chromosomal disorders. Aneuploidy is usually due to failure of segregation of chromosomes in meiosis I or meiosis II.[89]

Table 6: Examples of numerical aberrations (aneuploidies)

Aneuploidy	Karyotype	Incidence	Features
Down Syndrome Trisomy 21	47, XX or XY, +21	1/700 live births	Epicanthal folds, Hypotonia, Flat occiput
Edward syndrome Trisomy 18	47, XX or XY, +18	1/3000 live births	Rocker Bottom feet, Low set of ears Renal and cardiac anomaly
Patau syndrome Trisomy 13	47, XX or XY, +13	1/5000 live births	Microcephaly, Cyclopia, Heart defects
Klinefelter Syndrome	47, XXY (48XXXY; 49, XXXXY)	1/500 male births	Gynecomastia, Infertility
Turner Syndrome	45, X	1/5000 female births	Webbed neck Failure to mature sexually
Triple X Syndrome	47, XXX	1/1000 female births	Learning difficulties

Types of Mutations

A mutation is a change in the nucleotide sequence in coding portions of the DNA which may alter the amino acid sequences of proteins, or a change in noncoding regions of DNA which has the potential for changing expression of the gene. [90]

Mutations can also be categorized based on the function:[91]

1. The loss-of-function mutations cause a decrease or a loss of the gene product or the activity of the gene product.

2. The gain-of-function mutations cause an increase in the amount of gene product or its activity, leading to a toxic product responsible for a pathological effect.

With the development of new technologies, the detection of mutations has an increasingly central role in various areas of genetic diagnosis including preimplantation genetic diagnosis (PGD), prenatal diagnosis (PND), presymptomatic testing, confirmational diagnosis and forensic/identity testing.[92]

Methods used to detect the genetic changes in genetic clinics include:

- Conventional cytogenetics[93]
- Molecular cytogenetics (e.g., fluorescence in situ hybridization (FISH), multicolor FISH, locus-specific FISH; the polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative real-time RT-PCR, NGS (next generation sequencing), and microarray analysis.[94]

Conventional cytogenetics is the gold standard to evaluate chromosomal abnormalities, particularly structural and numerical chromosomal aberrations. The use

of molecular cytogenetic techniques, such FISH, to identify specific abnormalities has risen due to technical constraints like the necessity for fresh samples and challenges identifying masked or cryptic aberrations due to inadequate resolution by standard banding techniques.[95,96]

PCR (Polymerase Chain Reaction)

Kary B. Mullis created the ground-breaking technique known as PCR in 1983. The basis of PCR is the DNA polymerase's capacity to create new DNA strands that are complementary to the template strand.[97]

The Polymerase Chain Reaction (PCR) is a technique for replicating DNA that swiftly and precisely generates multiple copies of a particular DNA sequence.[98]

The Basics of PCR Cycling

The three main processes of PCR cycling reactions, which are repeated for 20 to 40 cycles. It is always carried out using an automated thermo cycler, which has the capacity to quickly heat and cool the reaction tubes.[99]

- Denaturation (95°C), 30 sec.
- Annealing (55–60°C), 30 sec.
- Extension (72°C), time depends on product size[100]

The target sequence of nucleic acid is denatured to single strands, primers specific for each target strand sequence are added, and DNA polymerase catalyzes the addition of deoxynucleotides to extend and produce new strands complementary to each of the target sequence strands (cycle 1). In cycle 2,

both double-stranded products of cycle 1 are denatured and subsequently serve as targets for more primer annealing and extension by DNA polymerase. After 25 to 30 cycles, at least 10^7 copies of target DNA may be produced by means of this thermal cycling.[101,102]

Application of PCR [(103-107)]

- PCR can be used for hereditary testing.
- PCR can be used for HIV testing. The purpose of PCR tests is to identify a single viral genome amid the DNA of more than 50,000 host cells.
- For genetic fingerprinting, a scientific technique that effectively distinguishes one person from the entire global population. Extraction of DNA from crime scene for convicted criminals.
- Parental testing (DNA sequencing) can benefit from the use of PCR for DNA fingerprinting.
- For DNA cloning.

MTHFR Gene

By catalysing the change from 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, methylenetetrahydrofolate reductase (MTHFR) plays a crucial part in the metabolism of folate and homocysteine. The C677T (thermolabile) allele and the A1298C allele are two frequent alleles of the MTHFR gene, which is found on chromosome 1 (1p36.3). Infants with C677T homozygosity have a slightly increased chance of developing neural tube abnormalities. Additionally, maternal C677T homozygosity seems to be a mild risk factor.[108]

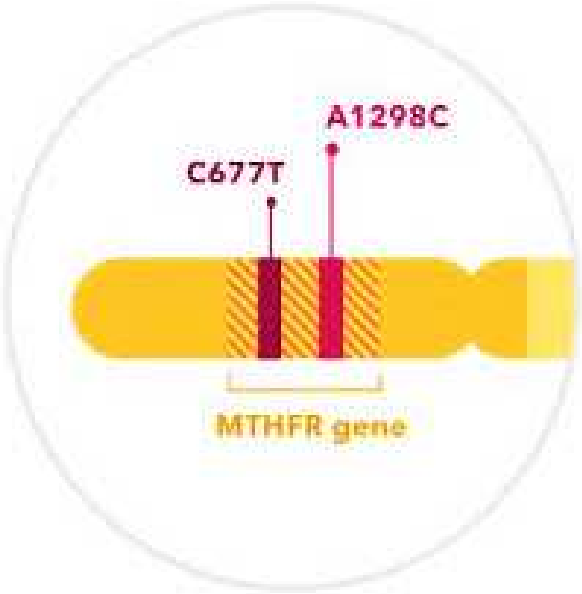


Figure 7: Location of MTHFR gene and mutations C677T and A1298C on chromosome 1.

A genetic variation, or single nucleotide polymorphism, called C677T or rs1801133, exists in the MTHFR gene. The MTHFR gene's position 677 indicates that "C" is the anticipated DNA nucleotide and "T" is a gene variation. The MTHFR gene is present in each person in two copies, one from the mother and one from the father. The MTHFR gene could have any of the following genotypes[109]:

- MTHFR 677 CC (two copies of C, one copy from each parent);
- MTHFR 677 CT (one copy of C from one parent, one copy of T from the other parent);
- MTHFR 677 TT (two copies of T, one copy from each parent).

Conditions associated with MTHFR gene

Anencephaly

Alzheimer's Disease

Cardiovascular Disease

Cleft Palate

Downs Syndrome

Schizophrenia

Thyroid Dysfunction

Depression

Diabetes

Fibromyalgia

Stroke

Miscarriage

METHODOLOGY

The present study was conducted by Department of Pathology, Jawaharlal Nehru Medical College Belagavi & Dr Prabhakar Kore Charitable Hospital & Medical Research Centre, Belagavi, Karnataka state.

The genetic study was conducted in Karnataka institute for DNA research (KIDNAR), Dharwad.

Study design

The study design was a cross sectional study.

Study period

The present study was conducted from January 2021 to December 2021.

Source of data

All stillborn fetuses, received at Department of Pathology, J N Medical College, for autopsy were included in the study.

For few cases, fetal cord blood was collected and formalin fixed placental tissue was sent for genetic analysis.

Sample size

A total of 160 fetuses were studied.

Total 20 samples were sent for genetic analysis.

Sampling procedure

Convenient sampling was done. All the samples which fulfilled both inclusion and exclusion criteria were studied.

Selection criteria

Inclusion

Fetuses received at the Department of Pathology for autopsy with proper consent were included in the study period.

Exclusion

Fetuses belonging to the parents who are not willing for the autopsy.

Ethical clearance

Prior to the commencement, ethical clearance for the study was obtained from Institutional Ethics Committee on Human Subjects Research, Jawaharlal Nehru Medical College, Belagavi.

Informed consent

The parents of stillborn fetuses and deceased neonates were briefed about the study and a written informed consent (Annexure I) was obtained.

Data collection

Prior to the autopsy a thorough external examination of the fetus was done and specific measurements were recorded. All the fetuses were evaluated using predesigned and pretested questionnaire for relevant history (Annexure II).

Autopsy Procedure

After receiving the fetus and the placenta, 10% formalin is injected into abdominal cavity, thorax (right and left side) and brain and it is stored in 10% formalin for 8-10 hours for fixation.

Facilities and equipment's

Access to photography setup and specimen x-ray machine was ensured. The entire body of the fetus was placed on the elevated dissection table to bring the work area up to chest level essentially under good lighting. In addition, availability of standard kilogram scale measuring device, a flexible rule and tape measure was confirmed. Other instruments of appropriate scale for the size of the body included pointed small scissors, scalpel, forceps and probes.

External Examination

- Body weight.
- Head circumference.
- Crown lump length
- Apparent gestation
- Maceration
- Meconium staining
- Demographic features, congenital malformation
- Other abnormalities (like edema).

Internal Examination

Comment on cranial, thoracic, and abdominal cavities.

Systematic description of major organs and tissues.

Placenta

Weight

Umbilical cord - Length, vessels, abnormalities.

Membranes - Complete, incomplete, colour, abnormalities, cut surface.

Histology

- At least one block of all major thoracic and abdominal organs (Right and left lungs, heart, liver, kidneys, adrenals and spleen)
- Adequate sampling of brain (One block from hind brain and one from hemispheres).
- Adequate sampling of placenta (Cord, membranes, focal lesions, grossly normal parenchyma.

The cause of death of stillbirth and early neonatal death was assigned by verbal autopsy by a single investigator. The factors contributing to the cause of perinatal deaths were noted. The cause of perinatal death was assigned by pathological autopsy and its correlation with verbal autopsy was noted.

Data Analysis

The obtained data is coded and entered in Microsoft Excel. Analysis is done using a SPSS 20 software. Descriptive statistics and chi-square test is used to evaluate the association between variables. The normal and abnormal distributive quantitative variable is compared using the students T-Test and the Mann-Whitney U test respectively. The results are given as the mean +/- Standard deviation, Median or Number percentage wherever appropriate. Data is analysed using Windows 10.

Genetic Analysis of rs1801133 SNP [C677T] of MTHFR gene :-

Materials and Methods:

1. Isolation of Genomic DNA from formalin fixed tissues:

The genomic DNA was isolated from formalin-fixed tissue samples using the phenol-chloroform method with slight modifications. The tissue samples were immersed in 1X GTE buffer [100mM Glycine, 10mM Tris HCl (pH 8), 1mM EDTA] for 12–72 hours to remove formalin completely, during which time the buffer was replaced with a fresh one every 3 hours. Then a small amount of tissue sample, around 25mg, was taken in a screw cap tube and washed with 100% ethanol, 70% ethanol, and distilled water for 1 minute, 3 minutes, and 5 minutes, respectively. For tissue digestion after complete removal of formalin, 500µl of alkali digestion buffer was added and autoclaved for 25 minutes at 120°C. The digested tissue was taken into another microcentrifuge tube and 500µl phenol: chloroform: isoamyl alcohol (25:24:1) was added, agitated for 5 minutes at room temperature, and centrifuged at 14000rpm for 5 minutes. The upper aqueous layer was taken into another tube to which 1 volume of chloroform was added, agitated for 5 minutes, and then centrifuged at 14000rpm for 5 minutes. The aqueous layer was taken after centrifugation, and 1 volume of isopropanol and 0.1 volume of 3M sodium acetate were added and mixed well, then centrifuged for 14000rpm for 30 minutes at room temperature. The pellet was then collected and washed with 70% alcohol, followed by centrifugation at 14000rpm for 5 minutes. After this, the pellet containing DNA was resuspended in T10E1 buffer.

2. Quality and quantity analysis of isolated products:

Agarose gel electrophoresis (0.8% agarose concentration) was performed to evaluate the quality of the DNA isolated from all tissue samples. A Nanodrop UV spectrophotometer was used to analyze the quality of isolated genomic DNA.

3. PCR amplification:

PCR was carried out for a total 10µl reaction mixture containing 1.5µl of DNA template, 1µl of 1X Taq buffer, 0.2µl of 100mM dNTPs, 0.2µl of 10 pM forward primer and 0.2µl of 10pM reverse primer, 0.06µl of Taq polymerase enzyme, 7.35µl of nano pure water and amplification performed under the cyclic conditions: 95ºC for 1 minute followed by 35 cycles of 95ºC for 30 seconds, 62.5ºC for 1 minute, 68ºC for 1 minute with a final extension at 68ºC for 5 minutes.

3.1 Agarose gel electrophoresis for PCR products: Visual Quality check for PCR products was performed using. 2.0% agarose gel prepared in 1X TAE buffer. Amplified DNA products were captured using a UV gel documentation system.

4. Sequencing of target SNP [rs1801133]:

The amplified product obtained was used for Sanger sequencing analysis. Cycle sequencing was carried out by using Big Dye terminator kit v3.1 (Applied Biosystem, USA) as per manufacturer guidelines. Sequencing of the target gene was performed using the direct Sanger sequencing method. The product was sequenced using ABI 3500 Sanger Sequencing platform and results were analysed on the DNA Sequencing Analysis Software v5.4 (Applied Biosystem, USA).

PHOTOGRAPHS

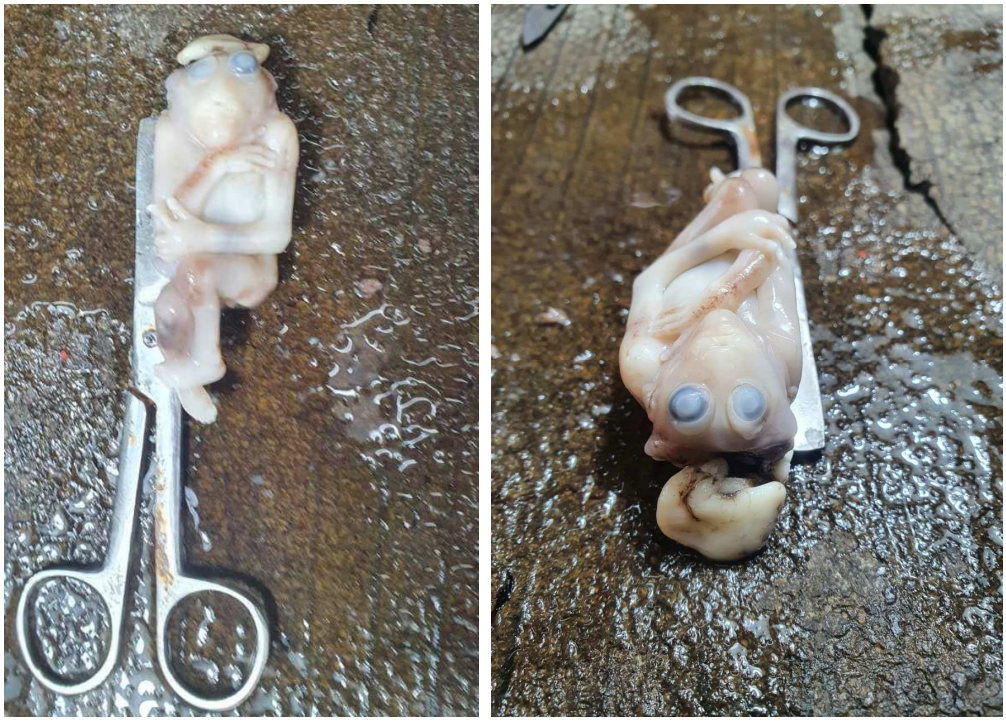


Figure 8- Fetus with anencephaly (Failure of cranial vault formation)

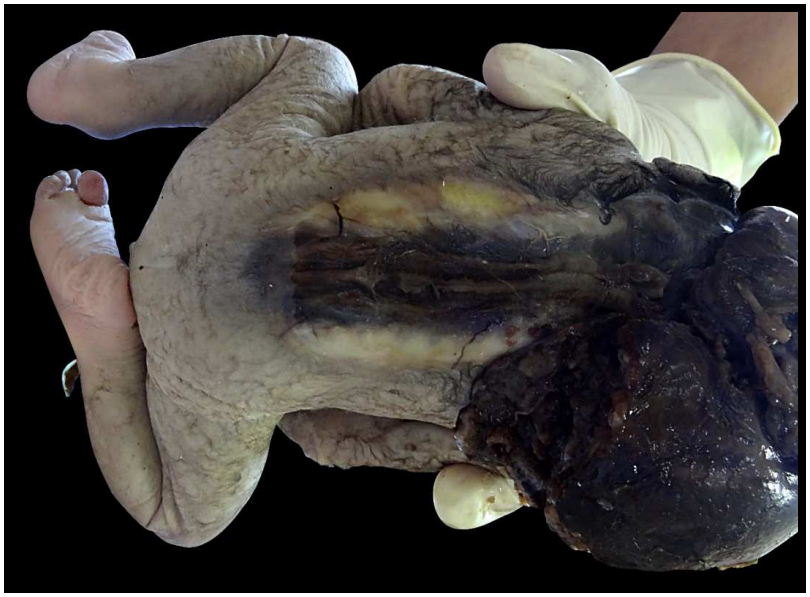


Figure 9- Anencephaly with Craniospinalrachischisis showing extension of the hairline to the mid thoracic level.

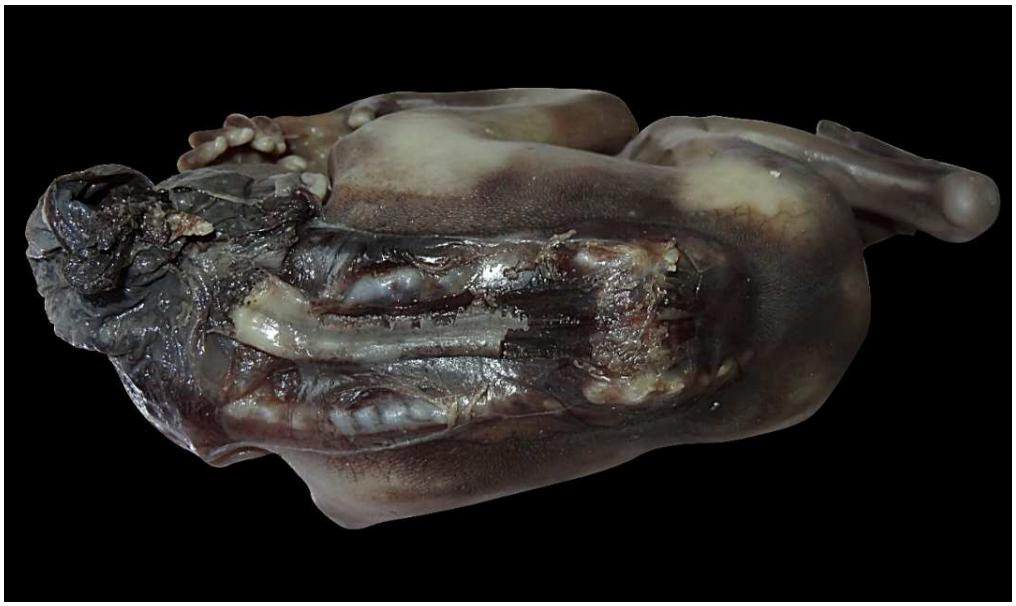


Figure 10 - Anencephaly with Spina Bifida

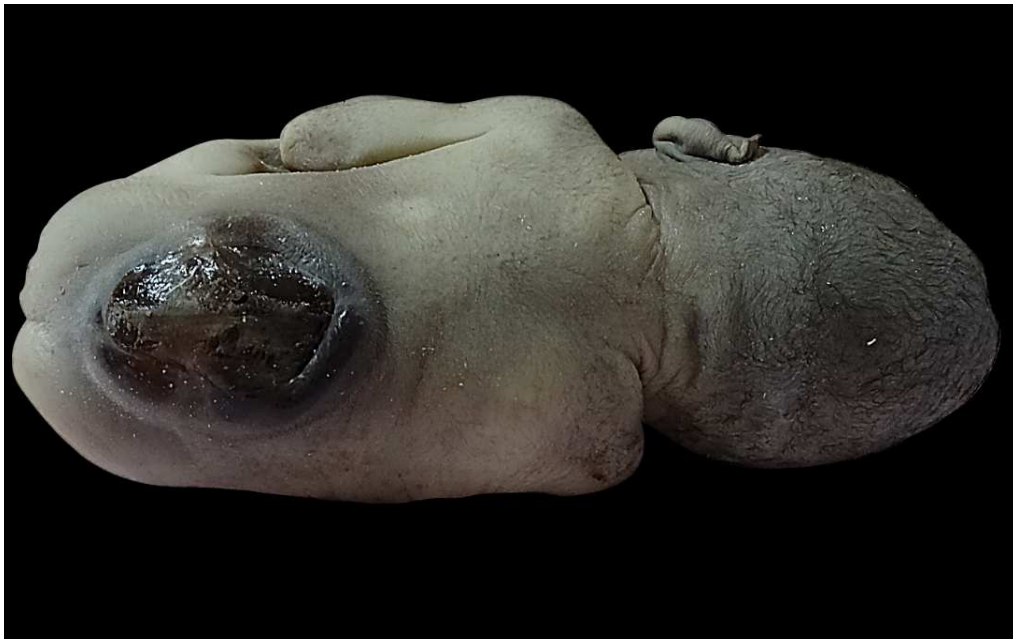


Figure 11– Meningomyelocele

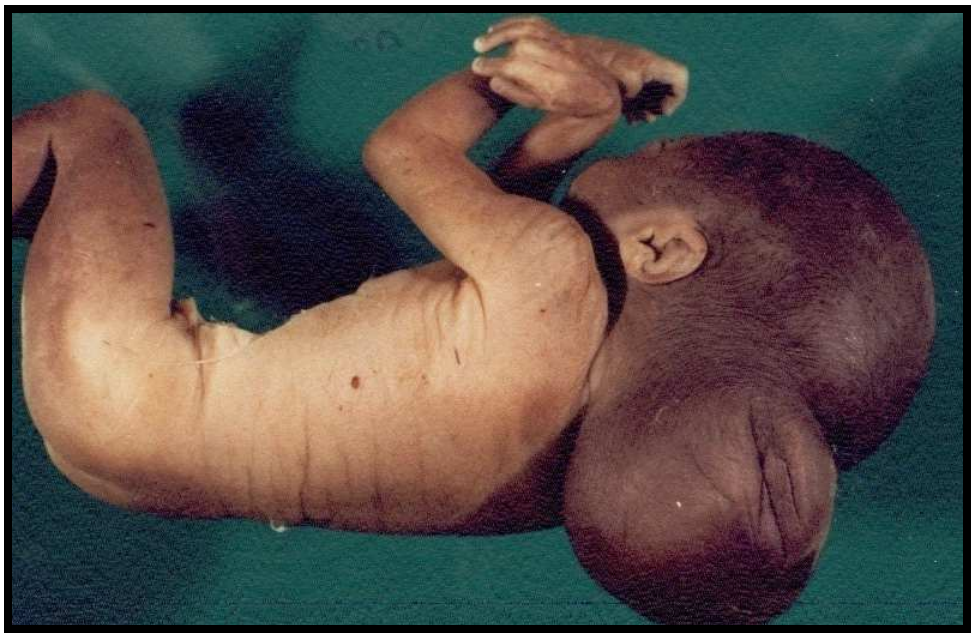


Figure 12 – Meningoencephalocele

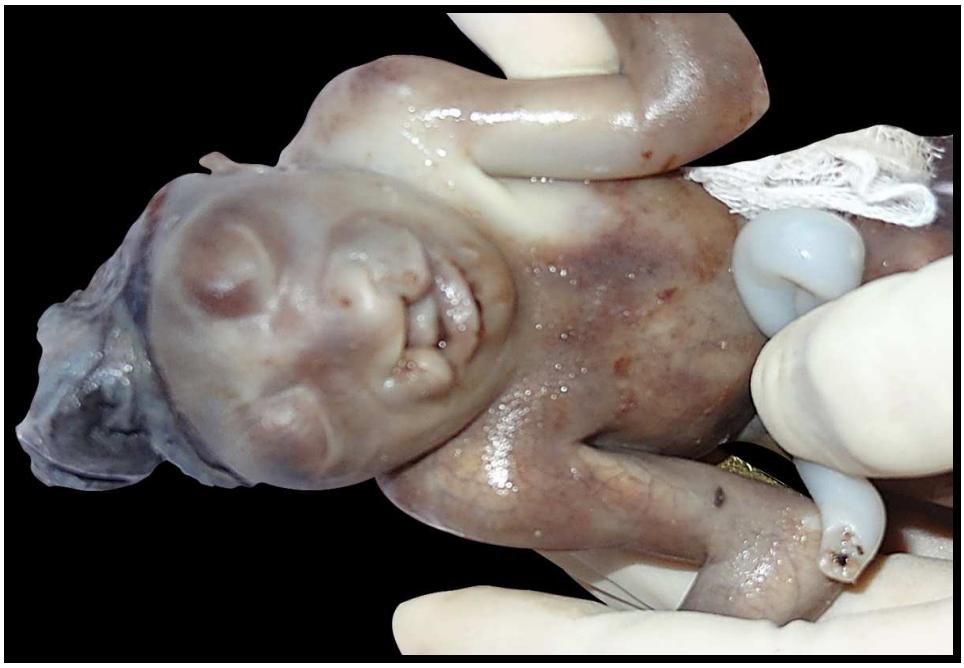


Figure 13- Bilateral cleft lip and cleft palate with anencephaly

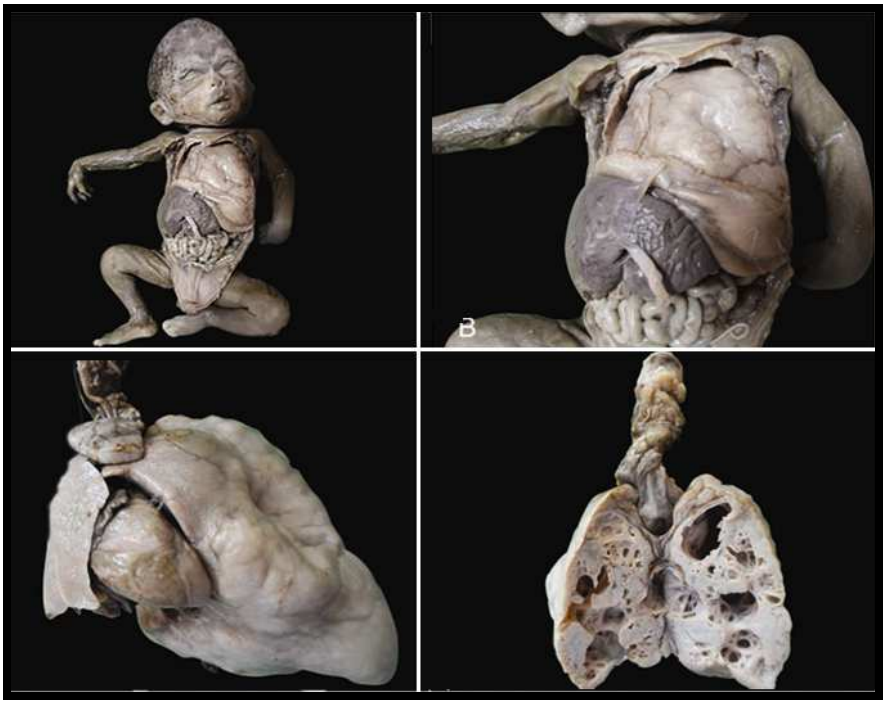


Figure 14- Congenital cystic adenomatoid malformation of lung:showing diffuse enlargement of lung with multiple cysts of varying sizes.

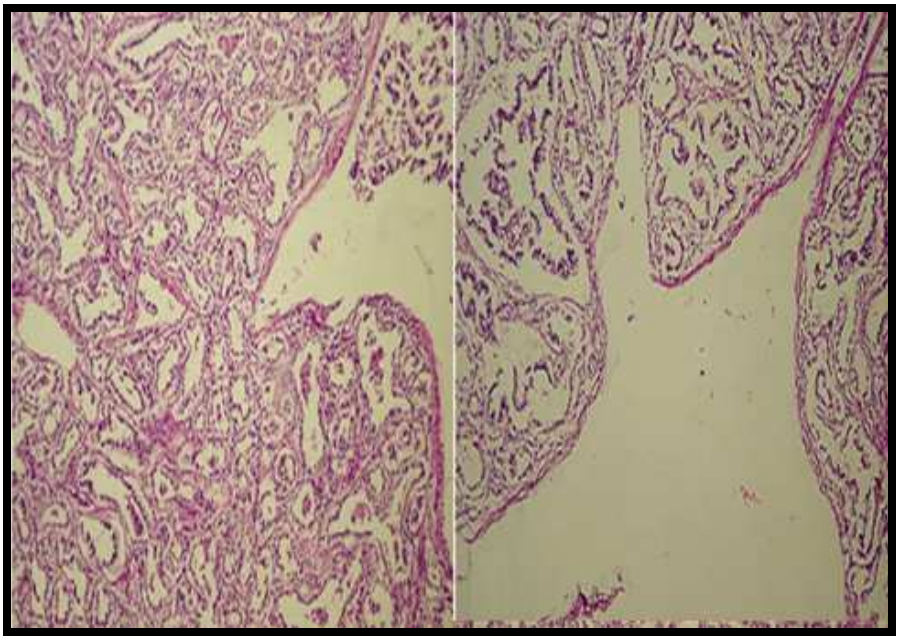


Figure 15 -Microscopy of Congenital cystic adenomatoid malformation cysts of varying sizes lined by tall columnar epithelium. (H & E,40X)

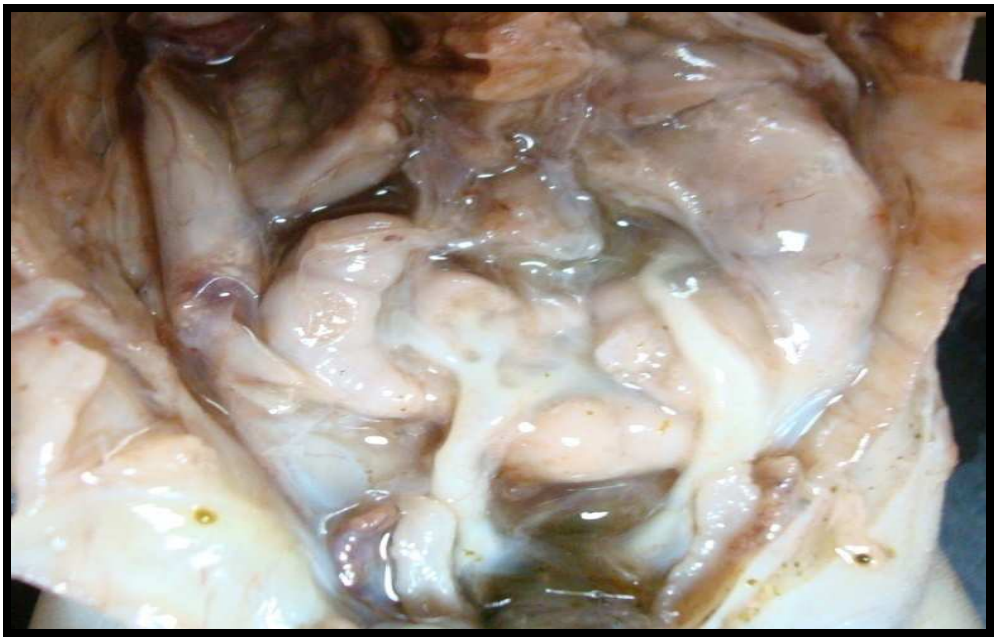


Figure 16 -Horse shoe kidney- kidneys are fused at the lower pole

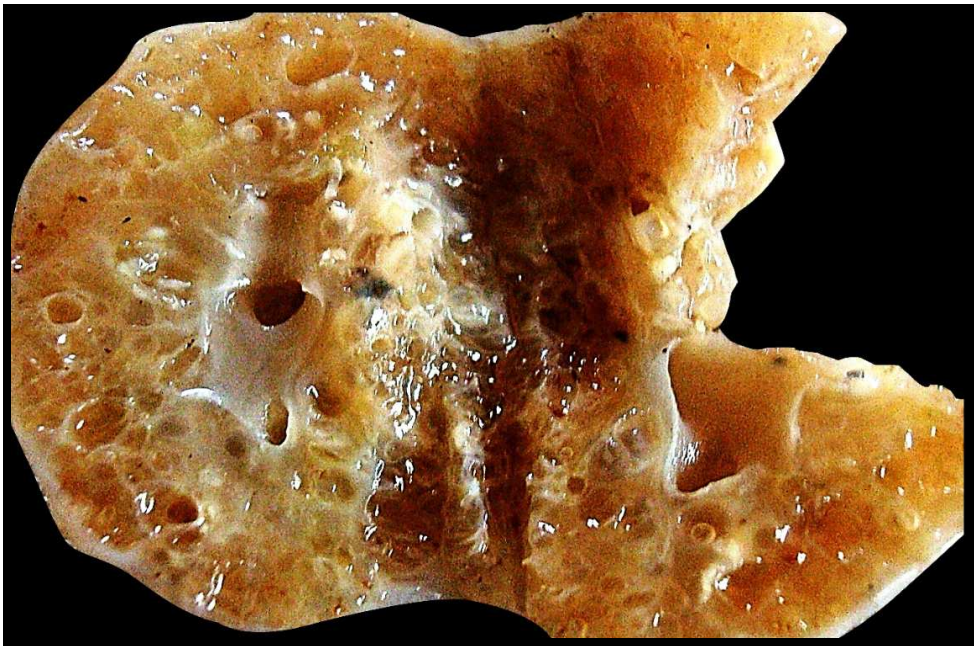


Figure 17 - Cut section of polycystic kidney showing cysts of different sizes.

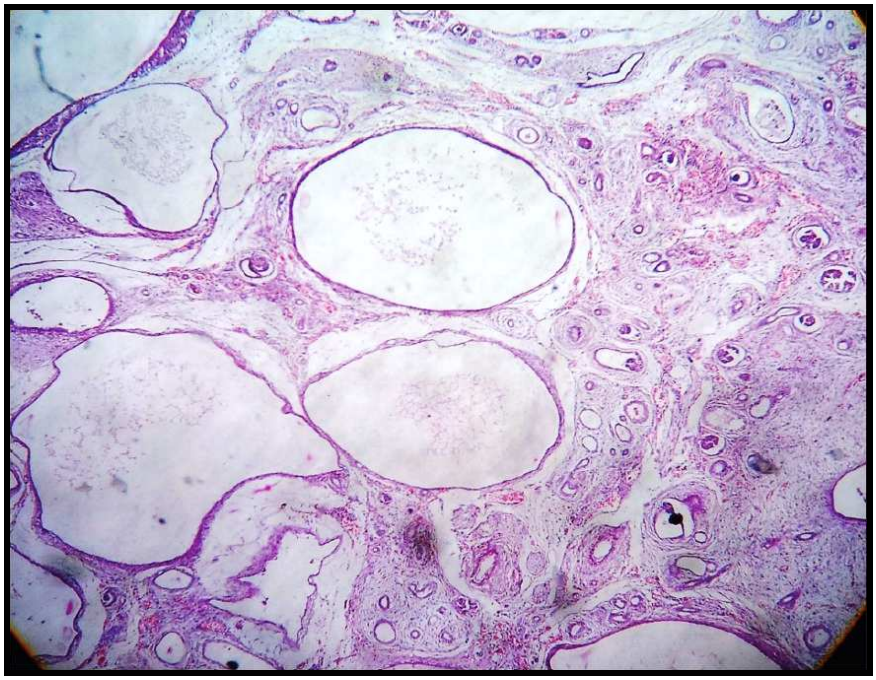


Figure 18: Photomicrograph showing cysts lined by flattened epithelium (PCKD), H&E, 40X

Figure 19- Absent anterior abdominal wall, evisceration of liver and intestinal loops in BSA (Body Stalk Anomaly)

Figure 20- Acalvaria with ill formed face in BSA

Figure 21 - Limb defect (Polydactly)

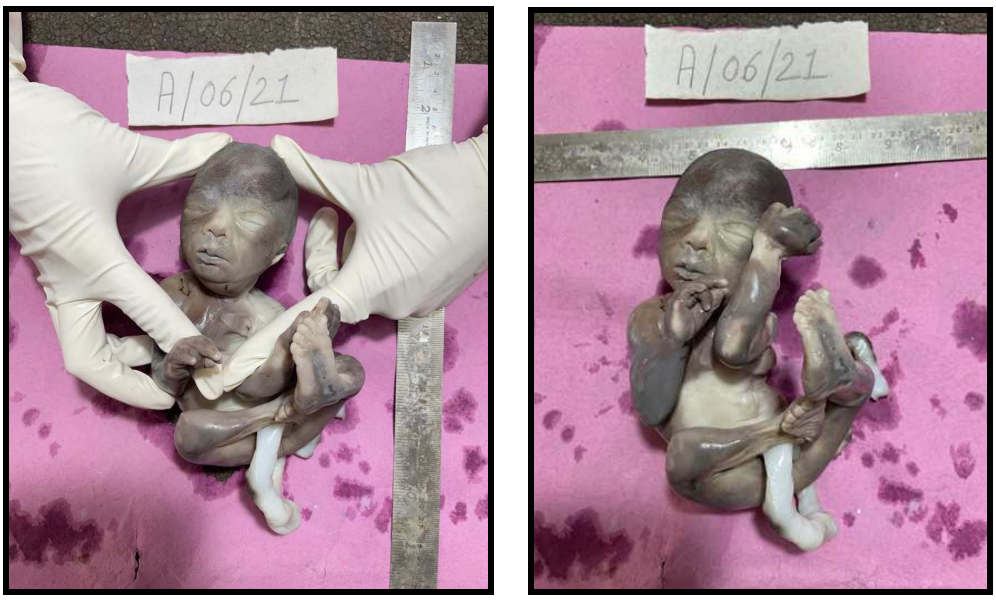


Figure 22 - Potters Facies (Showing depressed nasal bridge, prominent epicanthal folds, low set of ears)

Figure 23- Scoliosis and wrinkled skin in Potter's syndrome

RESULTS

The present cross-sectional study was conducted at Department of Pathology, Jawaharlal Nehru Medical College & Dr Prabhakar Kore Charitable Hospital & Medical Research Centre, Belagavi, Karnataka state, over a period of one year from 1st January 2021 to 31st December 2021.

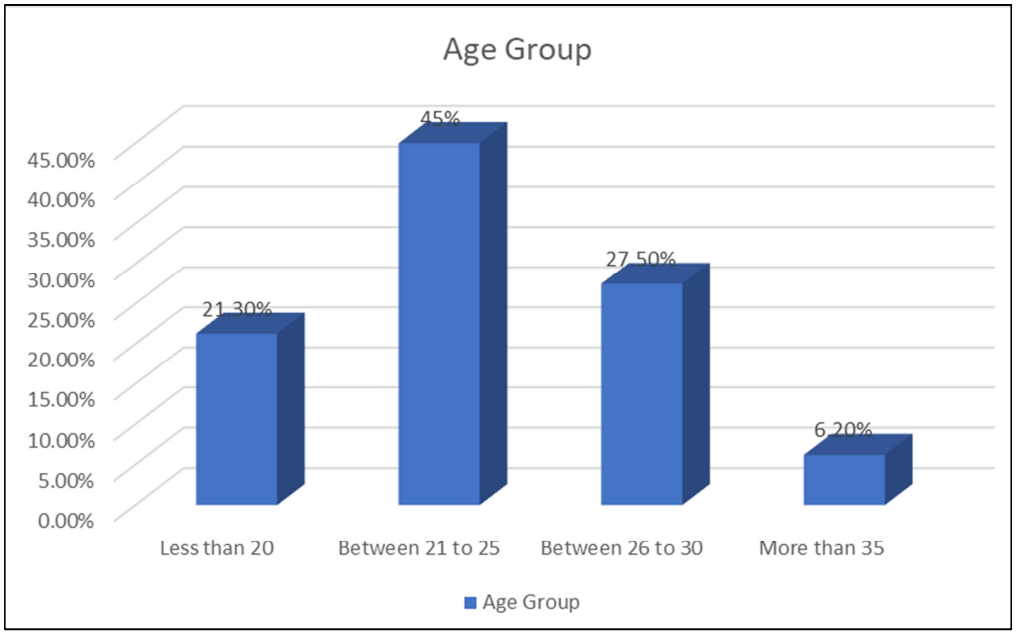
A total of 160 still born fetuses, received in the Department of Pathology for autopsy were included.

The data obtained were coded and entered into Master chart and analysed. The final observation and results were tabulated as below.

Table 7: Distribution of Mothers based on the age.

Age Group	Frequency (n=160)	Percentage
Less than 20	34	21.3
Between 21 to 25	72	45
Between 26 to 30	44	27.5
More than 35	10	6.2
Total	160	100

Graph 1: Graph wise distribution of mothers based on age

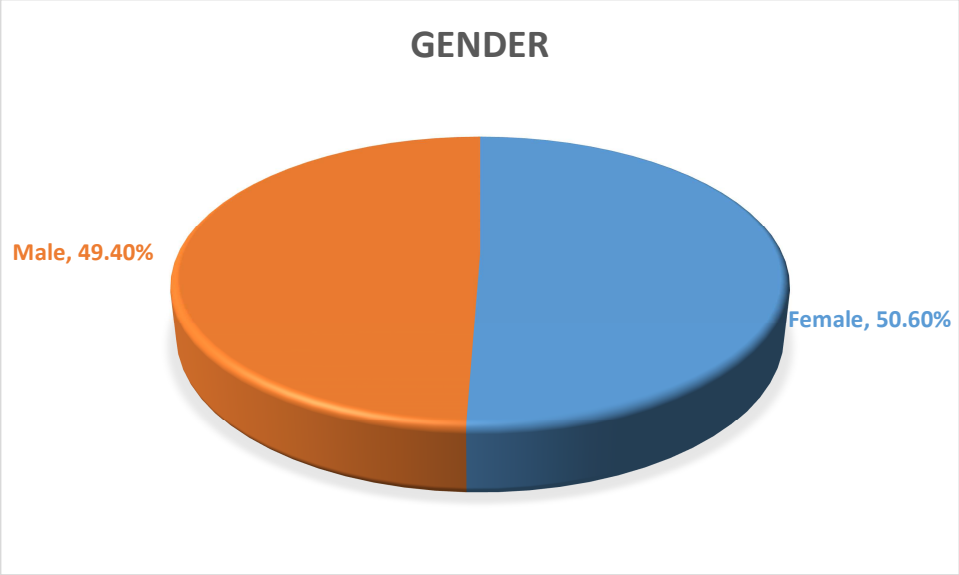


In present study, most of the mothers (45%) were aged between 21 to 25 years followed by 26 to 30 years (27.5%). The mean age of the mothers was 24.21+3.88 years of age. The median age was 24 years with range between 17 to 37 years.

Table 8: Distribution of fetus according to gender

GENDER	FREQUENYC(n=160)	PERCENTAGE
FEMALE	81	50.6
MALE	79	49.4

Graph 2: Graph wise distribution of fetus according to gender

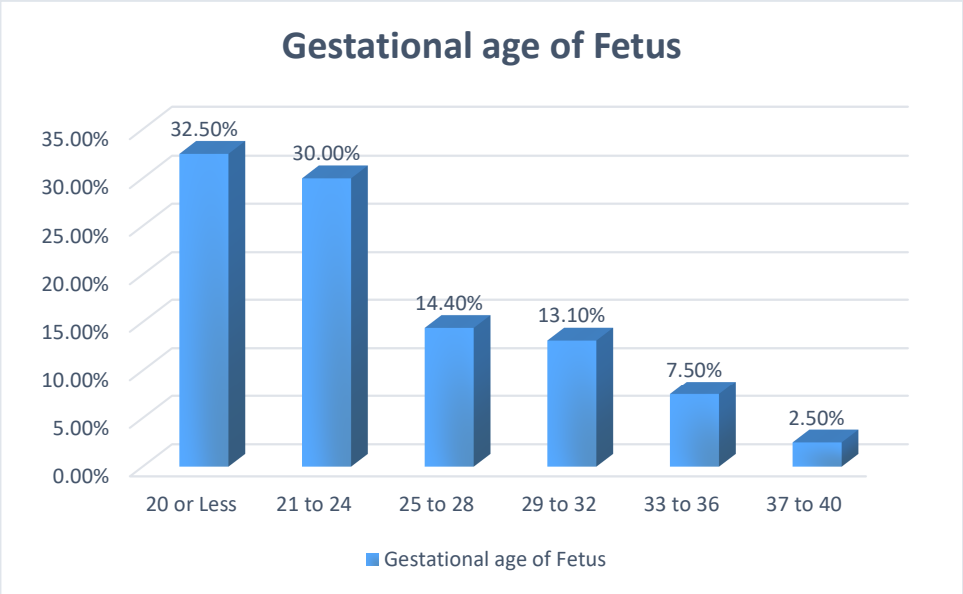


In this study 50.6% of fetuses were males and 49.4% were females.

Table 9. Distribution of fetus according to gestational age.

Gestational Age (Weeks)	Frequency(n=160)	Percentage
20 or less	52	32.5
21 to 24	48	30
25 to 28	23	14.4
29 to 32	21	13.1
33 to 36	12	7.5
37 to 40	4	2.5

Graph 3. Distribution of fetus according to gestational age

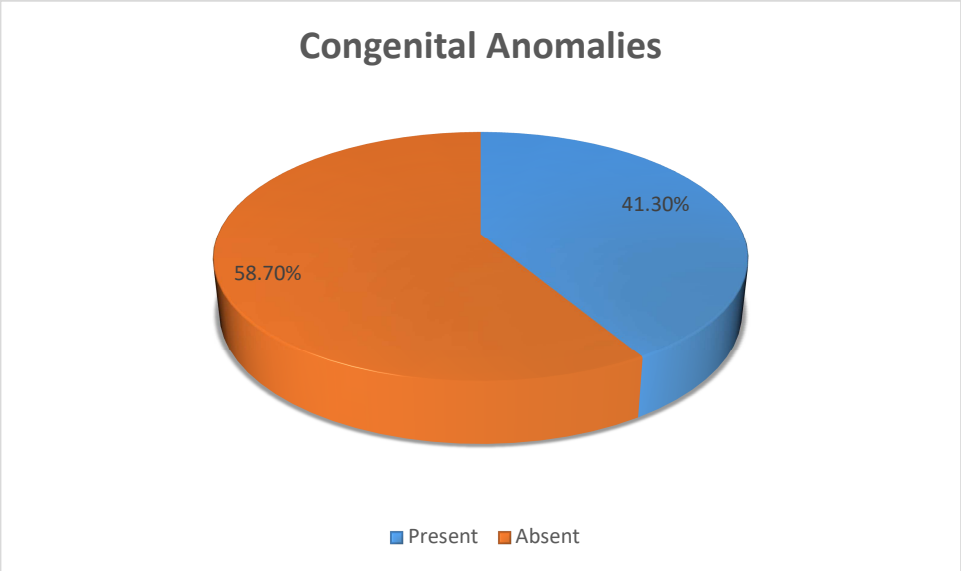


In the present study 52 (32.5%) fetuses had gestational age less than or equal to 20 weeks, 30% fetuses were in between 21 to 24 weeks of gestation and 14.4% fetuses had a gestational age between 25 to 28 weeks.

Table 10 : Distribution of study subjects based on congenital anomalies

Congenital Anomalies	Frequency(n=160)	Percentage
Present	66	41.3
Absent	94	58.7
Total	160	100.00

Graph 4 : Graph wise distribution of study subjects based on congenital anomalies

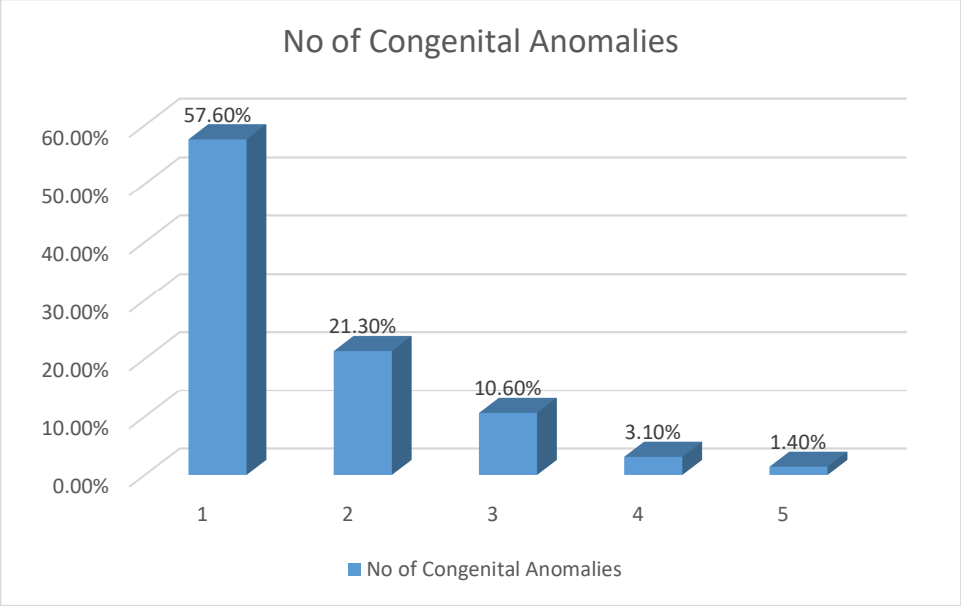


In this study, of 160 fetuses, congenital anomalies were found in 66 fetuses, hence the Prevalence of congenital anomalies was 41.3 %.

Table 11: Distribution of study subjects based on the number of congenital anomalies

No of Congenital Anomalies	Frequency(n=66)	Percentage
1	38	57.6
2	14	21.3
3	7	10.6
4	5	3.1
5	2	1.4
Total	66	100.00

Graph 5: Graph wise Distribution of study subjects based on the number of congenital anomalies

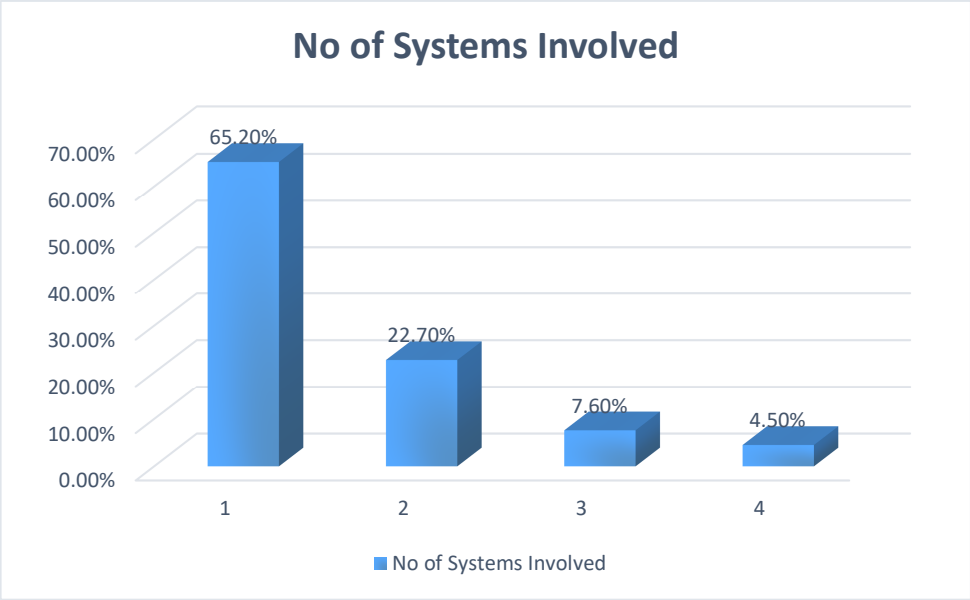


In this study, majority of fetuses (57.6 %) had only one anomaly, two anomalies were present in 14 (21.3%), three in 7 (10.6 %), four in 2 (1.4%), five in (3.1%) of fetuses respectively.

Table 12: Distribution of study subjects based on number of systems involved

Systems Involved	Frequency(n=66)	Percentage
1	43	65.2
2	15	22.7
3	5	7.6
4	3	4.5
Total	66	100.00

Graph 6: Graph wise distribution of study subjects based on number of systems involved



In this present study 43 (65.2%) fetuses had involvement of single system whereas, 15 (22.7%), 15 (22.7%), 5 (7.6%) and 3 (4.5%) had involvement of two, three, four systems respectively. Taking the above data into consideration, 43 (65.2%) fetuses had single system involvement and 23 (34.8%) fetuses had multisystem involvement.

Table 13: Distribution of subjects based on the congenital anomalies in different systems

Systems Involved	Frequency(n=66)	Percentage
Central Nervous System	24	36.4
Genitourinary Tract	18	27.3
GIT and Hepatobiliary	17	25.7
Musculoskeletal	9	13.6
Craniofacial	7	10.6
Respiratory System	5	7.6
Cardiovascular System	2	3.1
Miscellaneous	19	28.7

Graph 7: Graph wise distribution of subjects based on the congenital anomalies in different systems

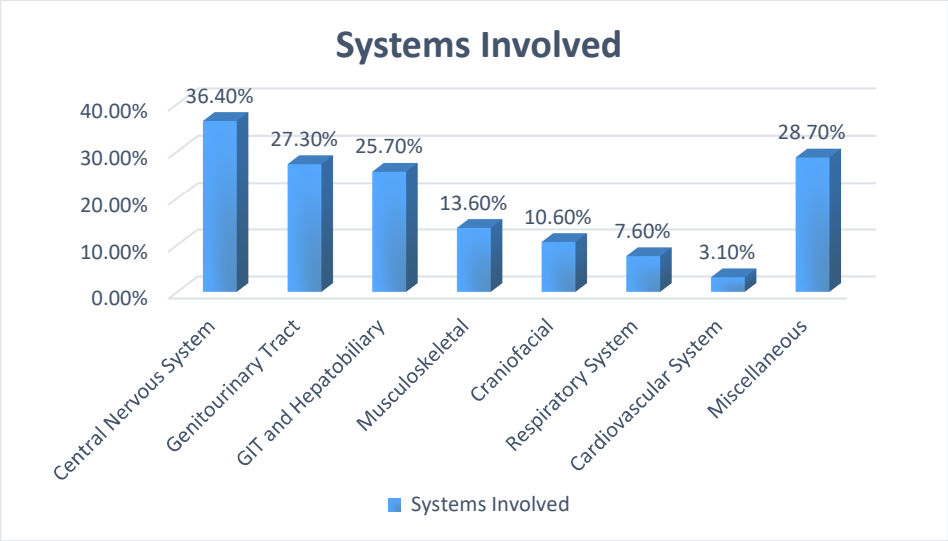
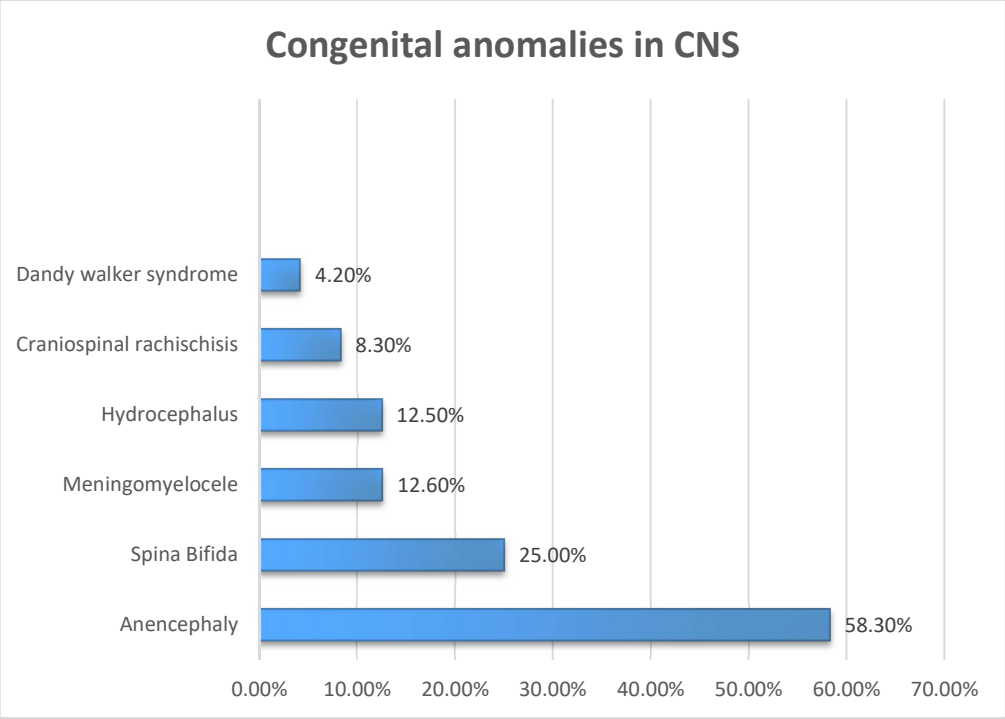


Table 7, and Figure 7, shows system wise distribution of congenital anomalies. The congenital anomalies were commonly seen in Central nervous system (36.4%).

Figure 14: Distribution of congenital anomalies in Central Nervous System

Congenital anomalies in Central Nervous System	Frequency(n=24)	Percentage
Anencephaly	14	58.3
Spina bifida	6	25.0
Meningomyelocele	3	12.6
Hydrocephalus	3	12.5
Craniospinal rachischisis	2	8.3
Dandy walker syndrome	1	4.2

Graph 8 : Graph wise distribution of congenital anomalies in Central Nervous System



In CNS, Anencephaly was the commonest congenital anomaly observed in 14 (58.3%) cases followed by spina bifida in 6 cases (25%), meningomyelocele in 3 (12.6%) cases hydrocephalus in 3 (12.5%) cases, and craniospinal rachischisis in 2 (8.3%) cases, dandy walker syndrome and meningoencephalocele was seen in 1 (4.2%) case.

In our study, Dandy walker malformatrion (DWM) was documented in one fetus. It is characterised as posterior fossa anomaly where there is hypoplasia or agenesis of vermis occurs and also cystic enlargement of the 4th ventricle which leads to upward displacement of tentorium and torcula. It has a prevalence of 1 in 35000 live births in the United States of America. The differential diagnosis of increased size of the cisterna magna can be made out by measuring the brainstem-vermian (BV) angle and brainstem-tentorium (BT) angle. The BV angle increases with severity and an angle below 18 degrees rate is considered as normal whereas, and angle of 18 to 30 suggest Blake's pouch malformation. The angle greater than forty five degree , strongly suggest Dandy Walker Malformation.[120]

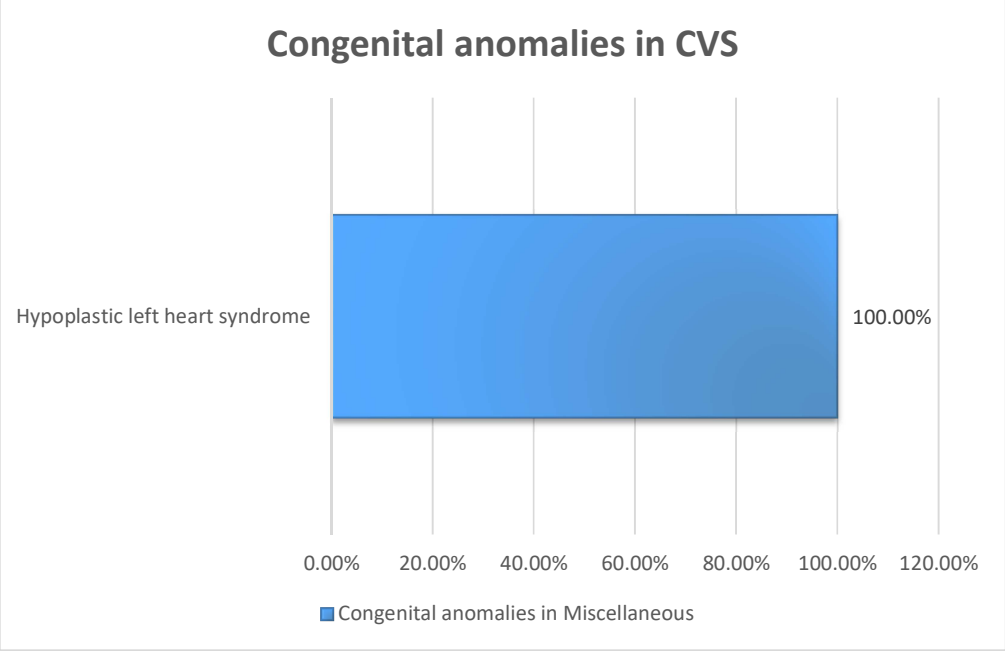
In this present case, the Magnetic resonance imaging of brain shows , cerebellar vermis hypoplasia, enlargement of the fourth ventricle, and supratentorial hydrocephalus.

The placental tissue was sent of this fetus for genetic analysis through PCR for MTHFR sequencing , which showed , 677 C>T (rs1801133) heterozygous mutation .

Table 15: Distribution of congenital anomalies in cardiovascular sysytem

Congenital anomalies in Cardiovascular System	Frequency (n=2)	Percentage
Hypoplastic left heart syndrome	2	100

Graph 9: Graph wise distribution of congenital anomalies in cardiovascular system

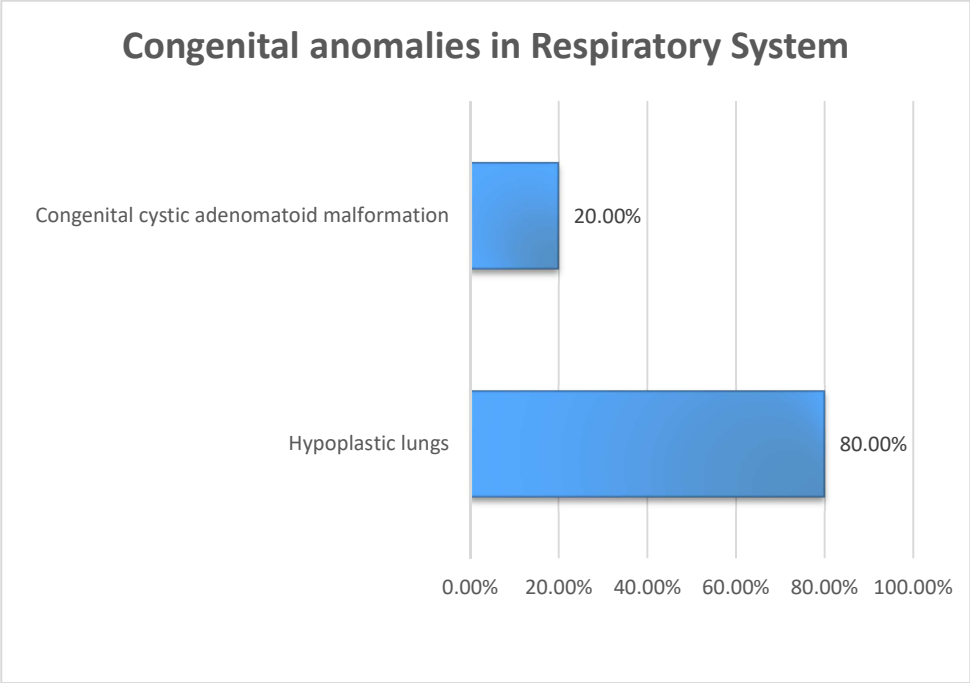


In the present study, with regard to cardiovascular system. Hypoplastic left heart syndrome was seen in one case.

Table 16: Distribution of congenital anomalies in Respiratory System

Congenital Anomalies in Respiratory System	Frequency (n=5)	Percentage
Hypoplastic lungs	4	80
Congenital cystic adenomatoid malformation	1	20

Graph 10 : Graph wise distribution of congenital anomalies in Respiratory System

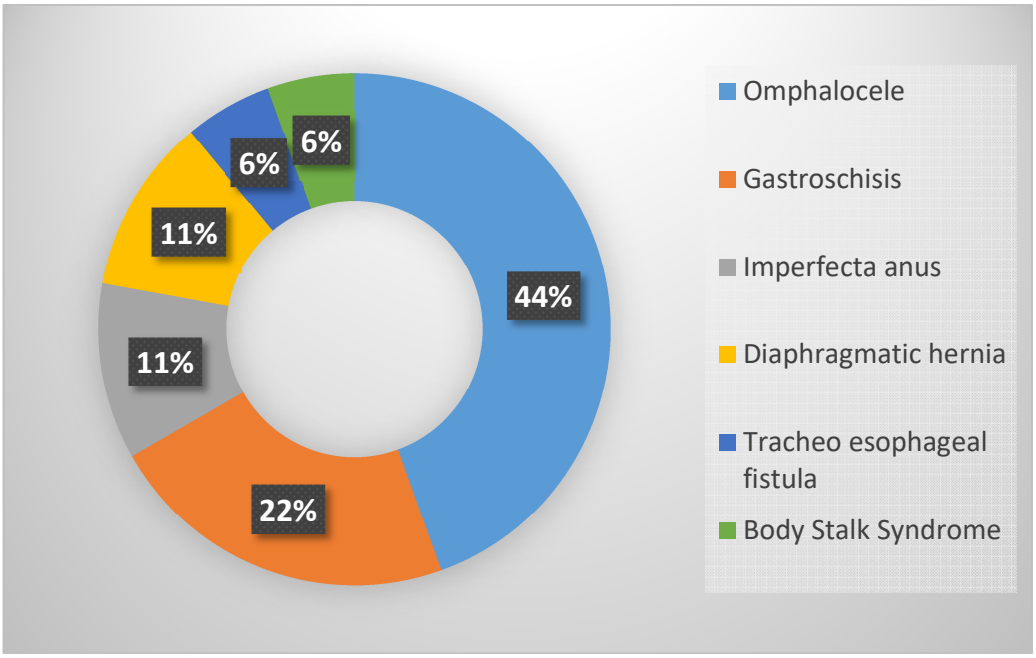


In respiratory system hypoplastic lungs were present in 4 cases (80%) followed by congenital cystic adenomatoid malformation in 1 (20%) case.

Table 17: Distribution of congenital anomalies in GIT and Hepatobiliary System

Congenital anomalies in GIT and Hepatobiliary	Frequency (n=18)	Percentage
Omphalocele	8	44.44
Gastroschisis	4	22.22
Imperfecta anus	2	11.11
Diaphragmatic hernia	2	11.11
Tracheo esophageal fistula	1	5.56
Body Stalk Syndrome	1	5.56

Graph 11: Graph wise distribution of congenital anomalies in GIT and Hepatobilliary system.



In Gastrointestinal tract , Omphalocele was the commonest seen in 8 (44.44%) cases followed by gastrochisis in 4 (22.22%) cases.

One case of body stalk anomaly was identified in the present study which is characterized by severe abdominal wall defect with evisceration of abdominal organs and associated with several other malformations of genitourinary tract, neural tube defects and various craniofacial malformations. The prevalence of this rare malformation has reported as 0.12 cases per 10,000 births (both live and still births).[120]

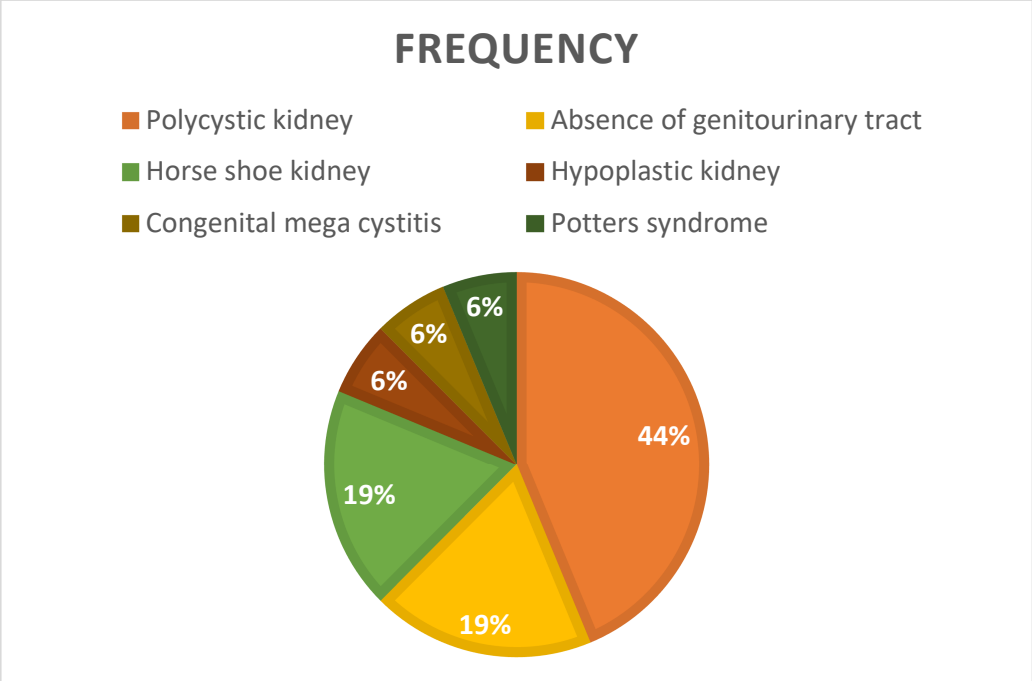
The present case fetus weigh 400gm with attached umbilical cord measuring 4cm and placenta weigh 70gm. On external examination, multiple anomalies were observed which were acalvaria, absent anterior abdominal wall with evisceration of liver and intestinal loops, gastroschisis, face was not developed, ambiguous genitalia, extradactly.

Microscopic finding revealed a single umbilical artery. The ultrasound finding revealed that cerebral parenchyma was thinned out and compressed. The cerebellum appear compressed against the occiput and a large abdominal wall defect was noted with herniation of viscera. A complete pathogenesis of this syndrome is not cleared but probable cause is believed to be an early rupture of amnion with direct mechanical pressure and amniotic band, or germinal disk abnormality, which represent failure of body folding in three dimensions.[119]

Table 18: Distribution of congenital anomalies in Genitourinary Tract

Congenital anomalies in Genitourinary Tract	Frequency (n=16)	Percentage
Polycystic kidney	7	44
Absence of genitourinary tract	3	19
Horse shoe shaped kidney	3	19
Hypoplastic kidney	1	6
Congenital Mega cystitis	1	6
Potter's Syndrome	1	6

Graph 12 : Graph wise Distribution of congenital anomalies in Genitourinary Tract



In the genitourinary tract system, Polycystic kidney was the commonest anomaly seen in 7 (44.4%) of cases. Absence of genitourinary tract in 3 (19%) , Horse shoe shaped kidney in 3 (19%) , hypoplastic kidney in 3 (19%) cases.

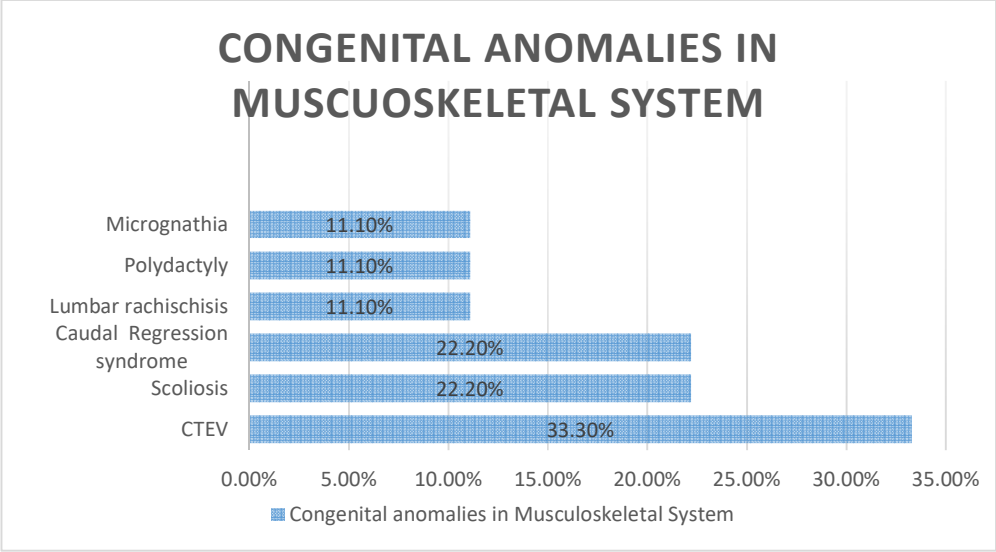
One rare case of congenital disorder of Potters's syndrome was also reported in the present study. This syndrome is characterised by typical physical appearance of the fetus, which is called as potter's facies , the cause of which is associated with pulmonary hypoplasia as a direct result of kidney failure. During 16 weeks of period of gestation, fetal urine is the largest source for amniotic fluid. In case of bilateral renal agenesis or obstruction in urinary tract can lead to reduce urine, production which can cause oligohydramnios.[122]

In this case the fetus on external examination presented with microganthia, epicanthal folds, flattened nose, cleft lip and wrinkled skin. On internal examination, there were urogenital dysplasia and cystic kidneys were noted with pulmonary hypoplasia and small sized heart. Potter's syndrome has an incidence 1 in 4,000 births, with a predominance in males as compared to females.[121]

Table 19: Distribution of congenital anomalies in Musculoskeletal System

Congenital anomalies in Musculoskeletal System	Frequency (n=9)	Percentage
Congenital talipes equinovarus	3	33.3
Scoliosis	2	22.2
Caudal Regression syndrome	2	22.2
Lumbar rachischisis	1	11.1
Polydactyly	1	11.1
Micrognanthia	1	11.1

Graph 13 : Graph wise Distribution of congenital anomalies in Musculoskeletal System

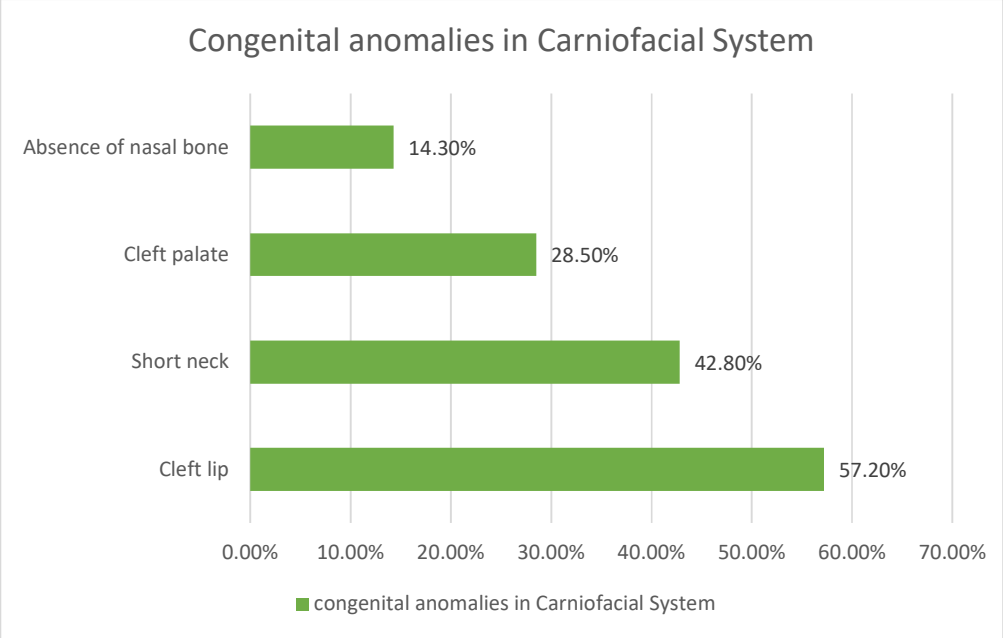


In this study CTEV was the commonest congenital anomaly seen in musculoskeletal system with 3 cases (33.3%). The other congenital anomalies observed in musculoskeletal system were scoliosis and caudal regression syndrome in 2 (22.2%) cases each.

Table 20: Distribution of congenital anomalies in Craniofacial System

Congenital Anomalies in Carniofacial System	Frequency(n=7)	Percentage
Cleft lip	4	57.2
Short neck	3	42.8
Cleft palate	2	28.5
Absence of nasal bone	1	14.3

Graph 14 : Graph wise distribution of congenital anomalies in Craniofacial System

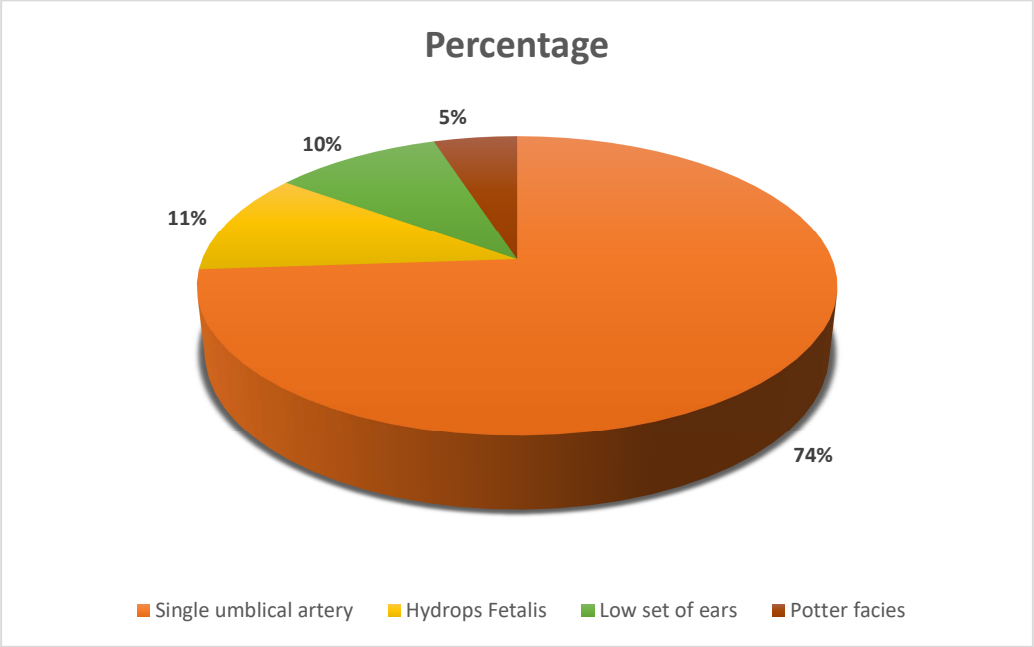


In the present study , congenital anomalies of the craniofacial system included cleft lip seen in 4 (57.2%) cases, short neck in 3 (42.8%) cases, Cleft palate in 2 (28.5%) cases and absence of nasal bone in 1 (14.3%) of cases.

Table 21: Distribution of congenital anomalies in Miscellaneous

Congenital anomalies in Miscellaneous	Frequency(n=19)	Percentage
Single umbilical artery	14	74
Hydrops Fetalis	2	11
Low set of ears	2	10
Potter facies	1	5

Graph 15 : Graph wise distribution of congenital anomalies in Miscellaneous



In Miscellaneous, Single Umbilical artery was commonest congenital anomaly, observed in 74% of cases followed by 11% cases of hydrops fetalis and 10% of Low set of ears, with 5% cases of potter facies .

Table 22 : Association of the Congenital Anomalies with the gender of the fetus

		Congenital Anomalies			
		Present(n=66)		Absent=(n=94)	
		Number	%	Number	%
Gender	Male	38	57.6	41	43.6
	Female	28	42.4	53	56.4
	Total	66	100.00	94	100.00

Chi Square = 3.023 , p= 0.08

In the present study, congenital anomalies were seen in 66 cases. In these 66 fetuses, 38 (57.6%) were male and 28 (42.4%) were females.

The difference was statistically not significant, p = 0.08.

Table 23 : Association of Mother Age with the congenital Anomalies

		Congenital Anomalies			
		Present(n=66)		Absent(n=94)	
		Number	%	Number	%
Age	Less than 20	15	22.7	19	20.2
	Between 21 to 25	26	39.4	46	48.9
	Between 26 to 30	22	33.4	22	23.5
	More than 30	3	4.5	7	7.4

Chi Square = 2.812 p=0.421

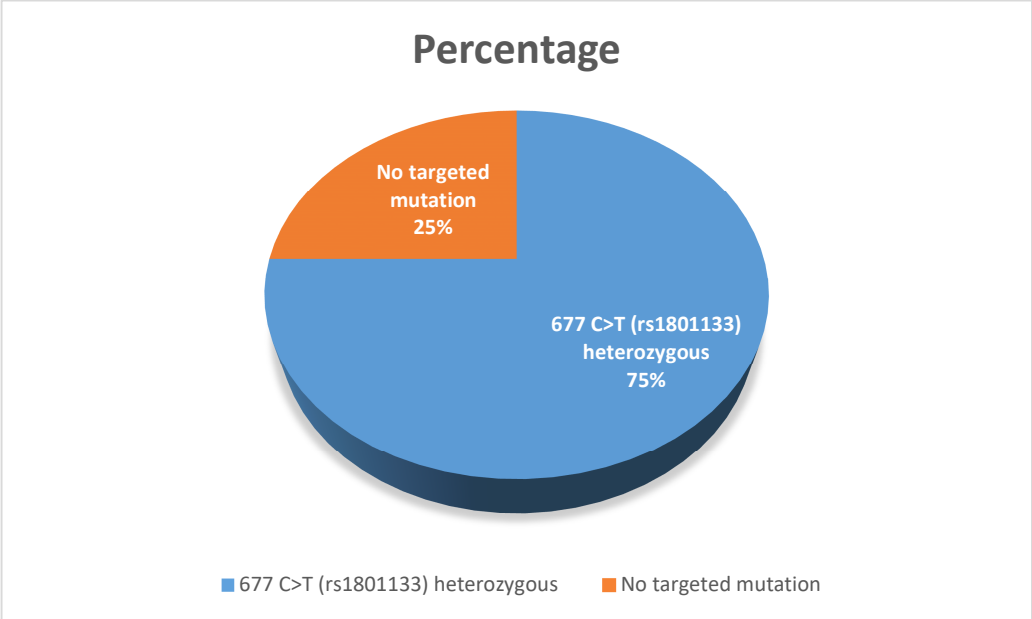
In the present study 72 mothers were aged between 21 to 25 years. Among these, congenital anomalies were seen in 39.40% and 48.90% did not have any congenital anomaly. However, no statistically significant difference was observed with the age of the mother and presence of congenital anomalies (p= 0.421).

Table 24. Sequencing results for MTHFR gene (rs1801133)

Sequencing result for MTHFR gene	Frequency(n=20)	Percentage
677 C>T (rs1801133) heterozygous	15	75
No targeted mutation	5	25
Total	20	100

In this present study, the sequencing result for amplification of targeted MTHFR gene was seen in 15 (75%) cases and 5 (25%) cases were not associated with targeted gene amplification.

Graph 16 : Sequencing result for MTHFR gene



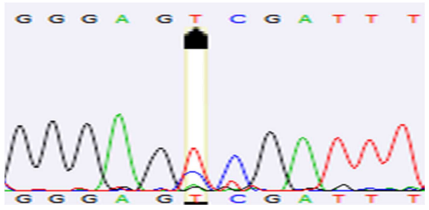
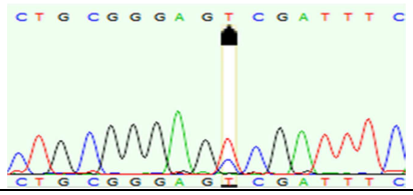
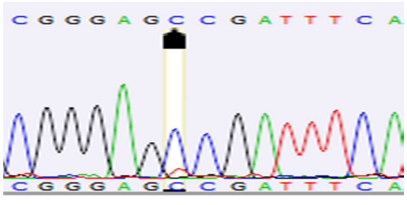
Genetics Result

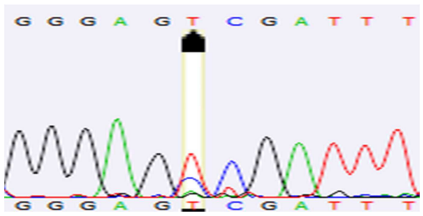
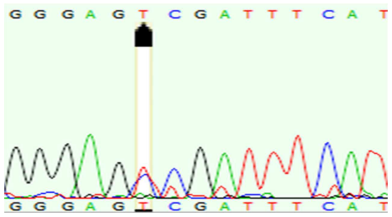
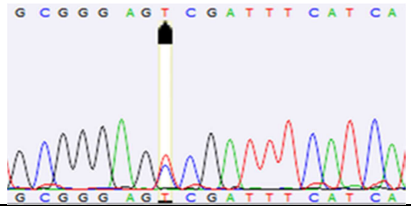
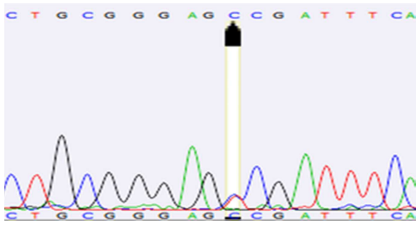
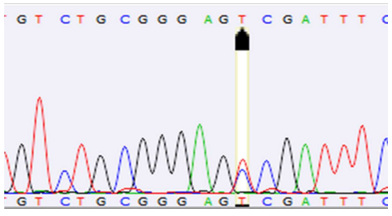
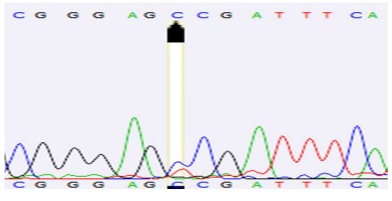
PCR amplification of Exon-5 of MTHFR gene for all the 20 samples has been performed successfully and the results were documented on 2% agarose gel electrophoresis. Amplified product of 202bp confirms the successful amplification of targeted region.

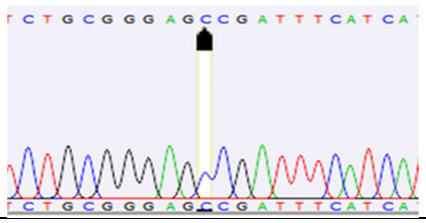
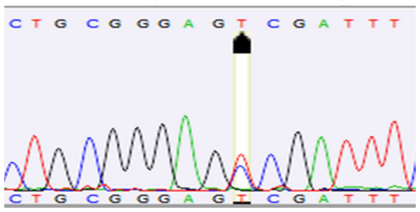
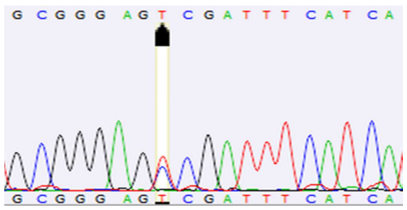
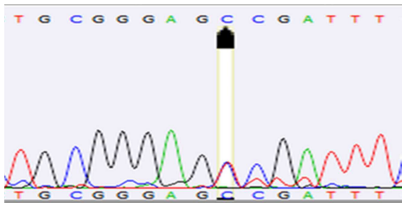
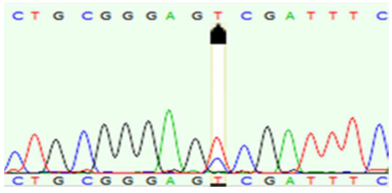
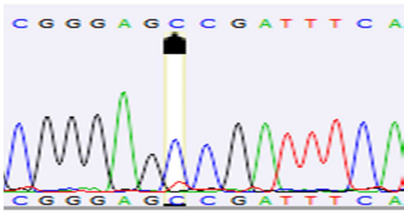
Sequencing analysis of rs1801133 SNP:

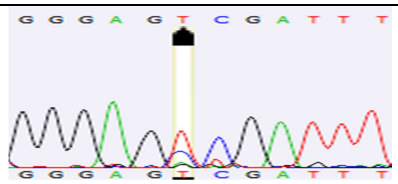
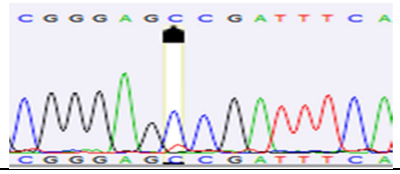
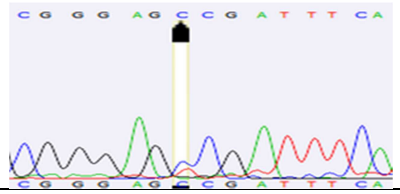
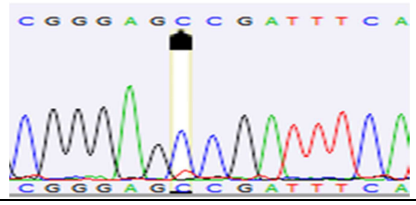
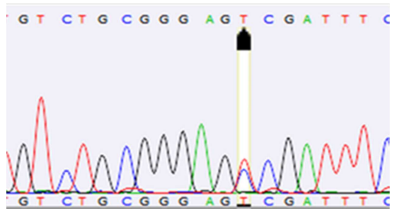
Sanger sequencing analysis of all the 20 samples for rs1801133 SNP revealed following results,

Table 25: Sequencing result and Chromatogram

SL.NO	AUTOPSY NO	DIAGNOSIS	SEQUENCING RESULTS	CHROMATOGRAM
1	A/136/21	Absent right kidney	677 C>T (rs1801133) heterozygous	
2	A/154/21	Multicystic dysplastic kidney	677 C>T (rs1801133) heterozygous	
3	A/17/21	Gastrochisis	No targeted mutation	

4	A/193/21	Hypoplastic uterus, Hypoplastic kidney, short stature, cystic hygroma, webbed neck, low set of ears	677 C>T (rs1801133) heterozygous	
5	A/100/21	Right kidney-Multicystic kidney	677 C>T (rs1801133) heterozygous	
6	A/111/21	Fetal dandy walker syndrome	677 C>T (rs1801133) heterozygous	
7	A/87/21	Craniospinal rachischisis	677 C>T (rs1801133) heterozygous	
8	A/61/21	Anencephaly	677 C>T (rs1801133) heterozygous	
9	A/06/21	Potters syndrome	677 C>T (rs1801133) heterozygous	

10	A/51/21	Congenital megacystitis	No targeted mutation	
11	A/75/21	Spina bifida with gastroschisis	677 C>T (rs1801133) heterozygous	
12	A/143/21	Anencephaly	677 C>T (rs1801133) heterozygous	
13	A/09/21	Anencephaly	677 C>T (rs1801133) heterozygous	
14	A/35/21	Neural tube defect	677 C>T (rs1801133) heterozygous	
15	A/47/21	Sickle cell crisis	No Targeted mutation	

16	A/54/21	Myelomeningocel-e	677 C>T (rs1801133) heterozygous	
17	A/163/21	Multicystic kidney disease	No targeted mutation	
18	A/189/21	Polycystic kidney, Polydactly	677 C>T (rs1801133) heterozygous	
19	A/124/21	Absence of genitourinary tract	No targeted mutation	
20	A/45/21	Meningomyelocele	677 C>T (rs1801133) heterozygous	

In our study, we have done genetic analysis of 20 fetal autopsy cases , the PCR amplification of Exon-5 of MTHFR gene for all the 20 samples has been performed successfully and the results were documented on 2% agarose gel electrophoresis.

The methylenetetrahydrofolate reductase protein chiefly reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid metabolic pathway, thus participating in purine synthesis, pyrimidine synthesis and DNA

synthesis *in vivo* as an indirect donor of methyl groups. The function of MTHFR protein is to maintain the effectiveness of one carbon cycle called as folate methionine cycle , which has role in DNA synthesis and DNA methylation. Any mutation in this gene will lead to increase in homocysteine levels which is harmful for the developing fetus. The most common single nucleotide polymorphism seen in this gene is 677C>T polymorphism where the alanine is replaced by valine and making this enzyme thermolabile, this reduces enzyme activity.[122]

A total of 20 fetal placental tissue and few cord blood was sent for identifying the polymorphism of MTHFR gene, and out of 20 samples, 15 samples came positive for polymorphism of MTHFR gene, confirming the 677 C>T (rs1801133) heterozygous variant and 5 had no mutation reported. These 15 fetal autopsy cases, included the anomalies mainly comprising of central nervous system like anencephaly, spina bifida, meningomyelocele, craniospinal rachischisis, dandy walker syndrome and urogenital anomalies, chiefly polycystic kidney disease.

In a study by Wang et al[123,124] on the association between 5, 10 – methylenetetrahydrofolate reductase and the risk of unexplained recurrent pregnancy loss in China, they have reported that all the C677T mutations of MTHFR were significantly associated with the risk of unexplained recurrent pregnancy loss (URPL) in the Chinese population. Another study by Radhika Kedar and Divya Chandel[126], on association of MTHFR C677T with Down syndrome, they have described a significant higher MTHFR C/T genotype in mothers of down syndrome children as compared to the mother's of normal children, suggesting that this polymorphism is an etiological risk factor for downs syndrome.

DISCUSSION

Fetal autopsy is performed by a specialist, perinatal pathologist, which is a fundamental tool in identifying the congenital malformations. The fetal autopsy plays an important role, not only in termination of pregnancy (TOP) for prenatally diagnosed fetus with anomalies but also in unexplained intrauterine death.[10]

Congenital malformations are due to result of multiple etiological factors, mainly the environmental and genetic factors. These malformations can present isolated or in combination, which can be identified by higher investigations, like imaging studies, fetal autopsy and genetic analysis. The fetal material should be stored for future genetic studies when autopsy fails in reaching to the diagnosis. Novel mutations and gene polymorphisms are discovered every year, therefore collecting tissue for genetic analysis is worthwhile.[15]

However, there is limited data on the incidence of congenital anomalies in fetal autopsy especially in India. Hence the present study was undertaken to determine the prevalence of congenital anomalies among fetal autopsies.

This cross-sectional study was conducted from 1st January 2021 to 31st December 2021 in Department of Pathology, Jawaharlal Nehru Medical College Belagavi & Dr Prabhakar Kore Charitable Hospital & Medical Research Centre, Belagavi, Karnataka state.

In this study, of the 160 fetuses, congenital anomalies were found in 66 fetuses. Based on these figures the prevalence of congenital anomalies was 41.3%. Mezawa H et al.[94] described a similar pattern of anomalies in his study where the

prevalence was 38.7% and in another study by Kasturilal et al[61] reported the incidence of about 32%. Quite recently a study from Turkey has described the higher incidence of fetal anomalies (58.39%).

In the present study, more than half fetuses (57.6%) had one anomaly, 21.3% had two anomalies and 10.6% fetuses had three anomalies. In the remaining four, five, anomalies were seen in 1.4% and 3.1% fetuses respectively. Several studies have reported the incidence of congenital anomalies, but they have not mentioned about the number of systems and anomalies involved in a single fetus hence the findings of the present study could not be compared.

In the present study, central nervous system was the commonest system involved in system wise distribution of congenital malformations, which was seen in 36.4% of fetuses followed by miscellaneous anomalies which were present in 28.7% fetuses. These results were quite analogous to the previous studies by Banerjee et al,[62] Nakamura et al,[69] Chopra et al[82] and S. P. Vinutha et al[127] , who have described the prevalence of CNS malformations in their study as 9%, 12.2%, 53.5%, and 34%. In numerous studies they have reported that the central nervous system was the predominant system involved for congenital malformations.

But the results of the current study differ from the earlier studies done by Banerjee et al[62] and Nakamura et al,[69] where they have described a lower incidence as compared to present study and in one of the other study done by Chopra et al[82] described a higher incidence rates. Another study[110] from Turkey has also reported a similar incidence of present study where the commonest system involvement was the central nervous system with incidence rate of 48.75%. In contrast to number of other

studies, which observed other system involvement like, cardiovascular and musculoskeletal system, as the commonest for congenital malformations.

Most significant yardstick for fetal anomalies since birth was the age of the mother.[111] For this reason, judicious examination of pregnant mothers above the age of 35 years is crucial, to avoid such pregnancies. In the present study, most of the mothers (45%) were aged between 21 to 25 years followed by 26 to 30 years (27.5%). The mean age was 24.62 ± 3.98 years. The median age was 24 years with range being 17 to 37 years. Of the 72 mothers aged between 21 to 25 years, congenital anomalies were seen in 41.02% (p=0.450).

In this study 50.6% of fetuses were females and 49.4% were males. In contrast to a recent 5-year study that found that congenital anomalies were more common in males (61.5%) than females (38.5%).[93] A recent study[110] done in Turkey described, the 60 cases with anomalies (37.5%) were females, 90 (56.25%) were males, and the sex of 10 cases (6.25%) could not be detected.

Among various studies on congenital anomalies, it has been observed to have a male preponderance.[113] However, in the present study, female preponderance was noted and sex predilection of malformation could not be done as the difference was statistically not significant (p=0.071). This could be attributed to the fact that the overall male to female ratio was 1:1. In a study of Padma S et al[15] from Hyderabad have mainly stressed on sex ratio in fetuses having congenital anomalies and observed that the occurrence was more in male babies as compared to female babies (1.7:1). An analogous ratios were also described in other studies done in India.[114] In an another study researchers have documented that the rate of malformations in males is approximately twice as that of females[115,116] whereas some of the studies have also

reported, that gender of the babies does not affect the prevalence of congenital anomalies and both genders have an equal distribution of malformations.[12] Only mild deviations in sex ratio have been documented for countless CAs, but so far no reasonable clarification for these aberrations has been found.[116] The implication of sex predominance can be corroborated by the fact that when there is a dominance of one sex for a specific malformation, this information can help predict the likelihood of the malformations in a patient and effect diagnostic approach.[117]

In CNS, the most common congenital malformation observed was anencephaly in 14 (58.30%) cases followed by spina bifida in 6 (25.00 %) cases and hydrocephalus in 3 (12.50%) cases. S. P. Vinutha et al[127] conducted a study on 50 stillborn fetuses, in the year 2021 where they had reported the majority of congenital abnormalities seen were CNS-related. The most frequent anomaly was meningomyelocele, followed by anencephaly. Comparable findings were recognized in various other studies also.[12]

In the present study, with regards to Cardiovascular system, hypoplastic left was present in one case. In a recent study[128] from India has described the most frequent anomaly was the ventricular septal defect. Tetralogy of Fallot, pulmonary stenosis, and an atrial septal defect were further abnormalities identified.

In this study in the fetuses with respiratory system congenital malformations, hypoplastic lungs were present in 4 cases (80.00%) followed by congenital cystic adenomatoid malformation in 1 (20.00%) case. In a study[15] from Turkey described, out of the four cases which had respiratory system anomalies, 2 cases had hypoplastic lungs, whereas diaphragmatic hernia and cystic adenomatoid malformation was seen in one case each.

In Gastrointestinal tract, Omphalocele was the commonest seen in 8 (44.44%) cases followed by gastroschisis in 4 (22.22%) cases. In contrast to one study[15] which has reported gastroschisis as being the commonest congenital anomaly present in 11 cases out of 15 cases of GIT congenital anomalies and others being, omphalocele (3 cases) and anal atresia (1 case).

In the genitourinary tract system, Polycystic kidney was the commonest anomaly seen in 7 (44.4%) of cases. Absence of genitourinary tract in 3 (19%) , horse shoe shaped kidney in 3 (19%) , hypoplastic kidney in 3 (19%) cases. Mohan H et al[33] and Banerjee CK et al[62] has also documented similar findings whereas another study[134] found hydronephrosis being the commonest anomaly, followed by cystic renal diseases and agenesis .

In this study CTEV was the commonest (33.20%) congenital anomaly seen in musculoskeletal system. The other congenital anomalies observed in musculoskeletal system were scoliosis and caudal regression syndrome in 2 (22.20%) cases each, followed by lumbar rachischisis, polydactyly and micrognanthia in one case each. In a study by Vandana Mudda et al[84], done in the year 2019 has reported achondrodysplasia in 2 cases out of 50 fetal autopsy.

In the present study congenital anomalies of the craniofacial system includes, cleft lip seen in 4 (57.2%) cases, short neck in 3 (42.8%) cases, cleft palate in 2 (28.5%) cases, cleft lip and palate in 6 (25%) cases . With regard to the miscellaneous congenital anomalies, single umbilical artery was seen in 13 (68.4%) cases, hydrops fetalis in 2 (10.50%) cases, potters facies in 1 (5.2%) cases.

SUMMARY

- A total of 160 stillborn fetuses were received at the Department of Pathology, J.N. Medical College, for autopsy was studied.
- Of the 160 fetuses studied, congenital anomalies were present in 66 fetuses. The prevalence of congenital anomalies was 41.3%
- Single congenital anomaly was seen in 57.6% fetuses whereas 42.4% fetuses had multiple congenital anomalies. Among those fetuses with multiple congenital anomalies, 21.3% had two anomalies and 10.6% fetuses had three anomalies.
- The central nervous system was the commonest system involved in 36.4% fetuses followed by miscellaneous system anomalies in 28.7%. In CNS, anencephaly was the commonest congenital anomaly observed in 14 (58.30%) cases.
- In the present study 72 mothers were aged between 21 to 25 years (45%). Among these, congenital anomalies were seen in 39.4% and 48.9% did not have any congenital anomaly. However, no statistically significant difference was observed with the age of the mother and presence of congenital anomalies ($p= 0.421$).
- In the present study congenital anomalies were seen in 66 cases. In these 66 fetuses, 38 (57.6%) were male and 28 (42.4%) were females. The difference was statistically not significant, $p = 0.08$.
- Three rare syndromes were also reported which were Dandy walker syndrome, Body stalk anomaly and Potter's syndrome.
- For genetic analysis of single nucleotide polymorphism in MTHFR gene, the direct Sanger sequencing method showed 677 C>T (rs1801133) heterozygous mutation in 15 cases out of 20 fetuses with congenital anomalies, proving that this mutation is an etiological risk factor for development of congenital malformations in fetuses and also a cause of adverse pregnancy outcome.

CONCLUSIONS

Congenital disorders are the major cause of new born deaths within the perinatal period, which can result in long-term disability with a significant impact on individuals, families, societies and health-care systems.

There is a lack of informative data about these malformations in India, a country that shares the maximum burden of neonatal mortality due to congenital birth defects

Good antenatal care can help in reducing these malformations in late pregnancy by timely detection and appropriate intervention. There should be more focus on pre-conceptional counseling. Moreover, these lethal malformations should be reported at pan-country level and a proper database about CMs should be established, which is lacking at present.

Furthermore, when these malformations are detected in late gestation, considering 100% mortality of the babies, proper counseling of the mother should be done. Few congenital malformations can be diagnosed prenatally with ultrasonography techniques, various maternal serum assays, but confirmation relies on actual examination of the fetus or neonate. These techniques cannot identify large proportion of congenital malformations.

Despite advances in imaging such as antenatal ultrasonography and serology, perinatal autopsy is superior and remains the gold standard investigation in diagnosing congenital malformations. The findings of autopsy are not only of theoretical importance but also of practical significance to clinicians, thus by a proper correlation and compilation of autopsy studies, prenatal USG, data documentation we

can counsel parents properly and predict the recurrence of anomalies in future pregnancies.

Genetic counseling provides information and support, assisting parents in making informed decisions. Through this process, parents learn about the risk of having a newborn with a congenital malformation and the nature of the disorder and its natural history, are advised on available testing for that particular case, and discuss options for risk management and family planning.

The direct benefits of autopsy to parents are not limited to refining the risk of recurrence. Even after autopsy, sometimes a definitive final diagnosis cannot be made and information given to parents may cover a range of possible diagnoses. In such cases the storage of fetal samples for possible future genetic analysis provides the hope of an accurate diagnosis (which may have ramifications for the wider family) at a much later date.

With the implementation of new genomic technologies in the diagnostic algorithm, like molecular karyotyping and next-generation sequencing that exceed conventional karyotyping and targeted molecular genetic testing with better diagnostic yield, approximately 50% of the genetic etiology of prenatally detected CAs can be explained. Therefore, we suggest a timely implementation of these technologies in prenatal diagnostics of CAs.

A wider importance of autopsy is in its value for quality control for prenatal diagnosis, teaching, and research purposes, therefore its is equally important to do genetic studies for further identifying and reducing the burden of congenital anomalies worldwide.

SCOPE AND LIMITATIONS

- In situations of prenatal abnormality of unclear origin and foetal death, foetal autopsy is routinely advised. Historically, autopsies were performed following terminations to ensure ultrasonography accuracy.

- Even though prenatal ultrasound can detect an early malformation, a detailed autopsy examination may add up the associated anomalies seen with that specific malformation.

- By identifying the prevalence of congenital anomalies, better measures can be taken for improving the antenatal care of the mother and introducing newer modalities like Next-generation sequencing, FISH, PCR etc. to detect these malformations early and reduce the recurrence risk. This also aid in providing better counselling to families who are dealing with a prenatally diagnosed foetal anomaly.

- Furthermore, because of financial and sample constraints, as well as a lack of infrastructure for genetic research, karyotyping of every anomalous fetus was not feasible in our study.

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ANNEXURE I – CONSENT FORM

Consent for the study "PREVALENCE OF CONGENITAL ANOMALIES IN FETUSES SUBJECTED FOR AUTOPSY- a hospital based cross- sectional study".

Purpose of the study:

Congenital malformations remain a common cause of perinatal deaths accounting for 10-15% in developing countries like India.

The purpose of this study is to determine the congenital anomalies in fetuses and to establish the clinical efficiency of fetal autopsy. Therefore, eventually helping the parents for future pregnancies and genetic counselling .

Authorization:

For the aim of this study , a written consent has to be provided by the parents/ guardians for granting permission to conduct a post mortem examination on the fetus. As a part of fetal autopsy, tissue samples (including those routinely processed for histology blocks and slides) and bodily fluids may be taken and used to determine the diagnosis and extent of disease. Photographs , X rays has to be taken in most cases and wherever possible genetic testing will be done in formulating a diagnosis and to aid the genetic counselling process.

Procedures involved :

During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge. If you

agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical history.

RISKS AND BENEFITS:

There is no increased risk involved in becoming a part of this study. The results derived at the end of study will benefit all similar patients.

VOLUNTARY PARTICIPATION / WITHDRAWAL FROM THE STUDY:

Taking part in the study is voluntary. You may choose not to enroll yourself in this study and may choose to leave the study anytime.

ALTERNATIVES:

Your decision regarding participation in study will not change present or future health care services offered to you at KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi. You would simply be excluded from the study if you wish to.

PRIVACY AND CONFIDENTIALITY:

All data collected or disclosed by you during the course of participation of study, will be kept fully confidential. If however during the course it becomes necessary for the progress of the course to disclose the identity, it would be done so only after your informed & written consent.

The only people who know that you are a research subject are members of the research team. No information about you will be disclosed to other without your written permission except following circumstances:

- In emergency to protect your rights AND welfare.
- If required by law.

AUTHORIZATION TO PUBLISH RESULT:

The results of the study may be used to publish an article. When the results of research published or discussed in a conference, no information will be displayed that would disclose your identity.

FINANCIAL INCENTIVES FOR PARTICIPATION:

No additional costs shall be incurred upon you for the purpose of this study.

It is purely being done with the idea of research and all the cost of study will be borne by the investigator.

QUESTIONS/CONTACT DETAILS:

If you have any queries about your rights as a study subject, you may call **DR. HARSHA HEGDE,** Chairperson, JNMC IEC & Scientist D, ICMR, National Institute Of Traditional Medicine, Belagavi. Phone number: 9480422500

CONSENT STATEMENT

I…….aged…….years exerting my free power of choice, hereby give my consent to do fetal autopsy and include the information obtained in the study - **"PREVALENCE OF CONGENITAL ANOMALIES IN FETUSES SUBJECTED FOR AUTOPSY"- A HOSPITAL BASED CROSS SECTIONAL STUDY.** I am aware that the data generated in the study will be utilised for research purposes. I confirm that I have not been offered any financial incentives to give the fetus for the study or I shall not derive any financial benefits from the study. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

I hereby give consent for the fetal autopsy and to utilize the information for the study.

NAME OF PARENT:

NAME OF WITNESS:

(SIGNATURE)

SIGNATURE/THUMB IMPRESSION OF PARENT:

DATE:

ANNEXURE II –

DATA COLLECTION PROFORMA

1. **Name: - B/O** **Ward/Unit**
2. **IP No:**
3. **Autopsy No:**
4. **Gestational Age**
5. **Sex:**
6. ☐ **MALE** ☐ **FEMALE**
7. **Received on:**

8. **Maternal History**

Age
Gravidity
Parity
Socioeconomic Status

- Number of family members
- Total income of the family

Family History

- Maternal history
- Paternal history

H/o Past Pregnancies

H/o Present pregnancy

9. **Antenatal History**

1. Toxemia of Pregnancy
2. Antepartum Hemorrhage
3. Placenta Previa
4. DM
5. Hydroamnios
6. Syphilis
7. UTI

8. TORCH Infection
9. Others
    - Antenatal Scanning, Investigations
    - Antenatal Vaccination
    - Obstetric History

10. <u>Fetal History</u>

Still born -
Macerated Still birth -
Birth weight -
Clinical features -
Birth injuries -
Fetal distress -
APGAR SCORE -
Clinical diagnosis -

11. <u>AUTOPSY NOTES</u>

External Examination

Weight
Measurements -

Crown heel length (CHL)
Crown rump length (CRL)
Head circumference
Chest circumference
Abdominal girth
Arm length
Leg length
Hand length
Foot length

Length of Umbilical Cord -
Natural orifices
Nose - Patent/ Discharge / Type of discharge / Anomalies
Ears - Patent / Discharge / Type of discharge / Anomalies
Mouth - Patent/ Discharge / Type of discharge / Anomalies
Anus: Patent/ Discharge / Type of discharge / Anomalies
Spine -
Skin –
External Anomalies – Present / Absent
Placenta -

Internal Examination

ON DISSECTION:

A. Central Nervous System
B. Respiratory System
C. Cardiovascular System
D. Gastrointestinal System
E. Hepatobiliary
F. Spleen
G. Pancreas
H. Genitourinary System and External Genitalia
I. Umbilical cord
J. Placenta
K. Membranes

MICROSCOPY:

A.Brain

a) Cerebrum
b) Cerebellum

B. Lungs
C. Heart
D. Liver
E. Spleen
F. Pancreas
G. Kidneys
H. Umbilical cord
I. Placenta
J. Membranes

12. Microscopic Diagnosis :

13. X- ray findings :

14. Clinico-pathological correlation :

15. Final diagnosis :

ANNEXURE III – KEY TO MASTERCHART

A	-	Anencephaly
Acal	-	Acalveria
AcLL	-	Accessory lobe of liver
AG	-	Ambiguous genitalia
AGT	-	Absence of genitourinary tract
ALLT	-	Aplasia of left lung
ANB	-	Absence of nasal bone
AUT	-	Absence of urinary tract
CCAM	-	Congenital cystic adenomatoid malformation
CFE	-	Crossed fused ectopia of kidney
CL	-	Cleft lip
CMK	-	Congenital megacystitis
CP	-	Cleft palate
CR	-	Craniospinal rachischisis
CRS	-	Caudal regression syndrome
CTEV	-	Congenital talipes equinovarus
CTJ	-	Craniothoracopagus Janiceps
Cy	-	Cyclopia
DH	-	Diaphragmatic hernia
DWS	-	Dandy walker syndrome
E	-	Encephalocele
Ea	-	Esophageal atresia
EK	-	Ectopic kidney
F	-	Female

GCK	-	Glomerulocystic change in bilateral kidneys
G	-	Gastroschisis
H	-	Hydrocephalus
HDF	-	Hydrops Fetalis
HSK	-	Horse shoe shaped kidney
HYLHS	-	Hypoplastic left heart syndrome
HyL	-	Hypoplastic lungs
Hyk	-	Hypoplastic kidney
HyS	-	Hypospadias
HyU	-	Hypoplastic uterus
La	-	Intestinal atresia
Lah	-	Imperfecta anus
I	-	Iniencephaly
It	-	Immature teratoma
LLF	-	Lower limbs fused together
LR	-	Lumbar rachischisis
LsE	-	Low set of ears
Mac	-	Macrocephaly
Mcl	-	Meckel's diverticulum
ME	-	Meningo encephalocele
Micg	-	Micrognanthia
Mic	-	Microcephaly
M	-	Male
MM	-	Meningomyelocele
O	-	Omphalocele

PcK	-	Polycystic kidney
Pf	-	Potters facies
P	-	Polydactyly
RBF	-	Rocker bottom feet
Rd	-	Renal dysplasia
RL2	-	Right lung two lobes
SA	-	Sacral agenesis
SB	-	Spina bifida
Scc	-	Sickle cell crisis
SH	-	Semilobar holoprosencephaly
SI	-	Situs inversus
SM	-	Sirenomelia
SN	-	Short neck
S	-	Scoliosis
SUA	-	Single umblical artery
ToF	-	Tracheo esophageal fistula
UUK	-	Unascended and unrotated kidney

ANNEXURE IV – KEY TO MASTERCHART

Serial number	Autopsy Number	Mother's Age (years)	Fetus age (weeks period of gestation)	Sex	CNS	CVS	RS	GIT and Hepatobiliary	GUT	Musculoskeletal	Craniofacial	Miscellaneous	Sequencing result for MTHFR gene
1	A/01/21	20	26	M	-	-	-	-	-	-	-	-	-
2	A/03/21	30	22	M	-	-	-	-	-	-	-	-	-
3	A/04/21	35	17	M	-	-	-	-	-	-	-	-	-
4	A/05/21	20	17	M	-	-	-	-	-	-	-	-	-
5	A/06/21	30	21	M	-	-	HyL	Iah	-	CTEV	-	Pf , S	-
6	A/07/21	29	28	M	-	-	-	-	-	-	-	SUA	-
7	A/08/21	23	18	M	A	-	-	-	-	-	-	-	-
8	**A/09/21**	20	23	M	A	-	-	G	AG	P	-	SUA	677 C>T (rs1801133) heterozygous
9	A/10/21	20	15	F	Acal	-	-	-	-	-	-	-	-
10	A/11/21	22	18	F	-	-	-	-	-	-	-	-	-
11	A/15/21	26	35	F	-	-	-	-	-	-	-	-	-
12	A/16/21	29	28	F	-	-	-	-	-	-	-	HdF , LsE	-
13	**A/17/21**	30	36	F	-	-	-	G	-	-	-	-	No targeted mutation
14	A/18/21	24	12	M	-	-	-	-	-	-	-	-	-
15	A/23/21	26	13	M	-	-	-	-	-	-	-	-	-
16	A/24/21	20	20	F	-	-	-	Iah	PcK	-	-	SUA	-
17	A/25/21	27	14	M	-	-	-	-	-	-	-	-	-
18	A/26/21	25	24	F	-	-	-	-	-	-	-	-	-
19	A/27/21	18	24	M	-	-	-	Iah	Pck	-	-	-	-
20	A/30/21	28	22	M	-	-	-	-	-	-	-	LsE	-
21	A/34/21	25	30	M	-	-	-	-	-	-	-	-	-
22	**A/35/21**	28	24	M	MM	-	-	-	-	-	-	-	677 C>T (rs1801133) heterozygous
23	A/36/21	34	18	F	-	-	-	-	-	-	-	-	-
24	A/37/21	25	19	F	-	-	HyL	-	-	-	-	-	-
25	A/40/21	20	20	F	-	-	-	-	-	-	-	-	-
26	A/41/21	25	13	F	-	-	-	-	-	-	-	-	-
27	A/42/21	18	17	F	-	-	-	-	-	-	-	-	-
28	A/43/21	19	13	M	-	-	-	-	-	-	-	-	-
29	**A/45/21**	20	13	F	MM	-	-	-	-	-	CL, CP	LsE, HdF	677 C>T (rs1801133) heterozygous
30	A/46/21	20	24	M	-	-	-	-	-	-	-	-	-
31	**A/47/21**	23	34	M	-	-	-	-	-	-	-	Scc	No targeted mutation
32	A/48/21	28	20	F	-	-	-	-	-	-	-	-	-
33	A/49/21	20	30	F	-	-	-	-	-	-	-	-	-
34	A/50/21	20	29	M	-	-	-	-	-	-	-	-	-
35	**A/51/21**	34	16	F	-	-	-	-	CMK	-	-	-	No targeted mutation
36	A/52/21	20	17	M	-	-	-	-	-	-	-	-	-
37	A/53/21	26	27	F	-	-	-	-	-	-	-	-	-
38	**A/54/21**	20	22	M	ME	-	-	-	-	-	-	-	677 C>T (rs1801133) heterozygous
39	A/55/21	24	32	F	-	-	-	-	-	-	-	-	-
40	A/56/21	20	18	M	-	-	-	-	-	-	-	SUA	-
41	A/57/21	26	19	F	-	HLHS	-	-	-	-	-	-	-
42	A/58/21	24	31	F	-	-	-	-	-	-	-	-	-
43	A/59/21	23	28	F	-	-	-	-	-	-	-	-	-
44	A/60/21	24	40	F	-	-	-	-	-	-	-	-	-
45	**A/61/21**	25	22	M	A, CR	-	HyL	-	-	-	-	-	677 C>T (rs1801133) heterozygous
46	**A/62/21**	23	22	M	A	-	-	-	-	-	-	-	677 C>T (rs1801133)

													heterozygous
47	A/63/21	32	24	M	CR	-	-	-	-	-	SN	-	-
48	A/64/21	28	20	F	-	-	-	-	-	LR	-	-	-
49	A/65/21	18	13	F	-	-	-	-	-	-	-	-	-
50	A/66/21	23	18	F	-	-	-	-	-	-	-	-	-
51	A/67/21	22	35	F	-	-	-	-	-	-	-	-	-
52	A/68/21	24	24	M	-	-	-	-	-	-	-	-	-
53	A/70/21	28	18	F	-	-	-	-	-	-	-	-	-
54	A/71/21	32	21	F	-	-	-	-	-	-	-	-	-
55	A/73/21	22	20	F	-	-	-	-	-	-	-	-	-
56	A/74/21	20	23	F	-	-	-	-	-	CRS	-	-	-
57	**A/75/21**	30	13	M	SB	-	-	G	-	SM	-	-	677 C>T (rs1801133) heterozygous
58	A/76/21	25	20	F	-	-	-	-	-	-	-	-	-
59	A/77/21	30	22	F	-	-	-	-	-	SA	-	-	-
60	A/79/21	27	17	M	-	-	-	-	-	-	-	-	-
61	A/80/21	28	13	M	-	-	-	-	-	-	-	-	-
62	A/82/21	28	28	M	-	-	-	-	-	-	-	-	-
63	A/84/21	22	16	M	-	-	-	-	-	-	-	-	-
64	A/86/21	22	26	F	-	-	-	-	-	-	-	-	-
65	**A/87/21**	24	18	M	CR	-	-	-	-	-	-	-	677 C>T (rs1801133) heterozygous
66	A/88/21	26	22	M	-	-	-	-	-	-	-	-	-
67	A/89/22	26	32	F	-	-	-	-	-	-	-	-	-
68	A/92/21	19	22	M	-	-	-	-	-	-	-	-	-
69	A/93/21	22	14	M	A	-	-	-	HSK	-	-	-	-
70	A/94/21	22	15	F	-	-	-	-	-	-	-	-	-
71	A/95/21	27	24	M	-	-	-	-	-	-	-	SUA	-
72	A/97/21	26	19	M	-	-	-	-	-	-	-	-	-
73	A/98/21	25	18	M	-	-	-	-	-	-	-	-	-
74	A/99/21	23	27	F	-	-	-	-	-	-	-	-	-
75	**A/100/21**	26	23	M	-	-	-	-	PcK	-	-	-	677 C>T (rs1801133) heterozygous
76	A/105/21	18	32	F	-	-	-	-	-	-	-	-	-
77	A/106/21	24	16	F	-	-	-	-	-	-	-	-	-
78	A/107/21	18	20	M	-	-	-	-	-	-	-	-	-
79	A/108/21	25	22	F	-	-	-	-	-	R	-	SUA	-
80	A/109/21	23	17	M	-	-	-	-	-	-	-	-	-
81	A/110/21	27	38	M	-	-	-	-	-	-	-	-	-
82	**A/111/21**	23	22	M	DWS	-	-	-	-	-	-	-	677 C>T (rs1801133) heterozygous
83	A/113/21	22	21	M	-	-	-	-	-	-	-	-	-
84	A/116/21	23	34	M	-	-	-	-	-	-	-	-	-
85	A/118/21	21	24	M	-	-	-	-	-	-	-	-	-
86	A/119/21	24	34	F	-	-	-	-	-	-	-	-	-
87	A/120/21	21	19	F	-	-	-	-	-	-	-	-	-
88	A/122/21	35	24	F	-	-	-	-	-	-	-	-	-
89	A/123/21	20	29	F	-	-	-	-	-	-	CL	-	-
90	**A/124/21**	19	23	M	-	-	-	-	AGT	-	-	SUA	No targeted mutation
91	A/125/21	25	15	F	-	-	-	-	-	-	-	-	-
92	A/126/21	24	19	M	-	-	-	-	-	-	SN	-	-
93	A/127/21	21	20	F	-	-	-	-	-	-	-	-	-
94	A/129/21	24	26	F	-	-	-	-	-	-	-	-	-
95	A/131/21	27	27	M	-	-	-	-	-	-	CL , ANB	-	-
96	A/132/21	22	32	M	-	-	-	-	-	-	-	-	-
97	A/133/21	32	24	F	-	-	-	-	-	-	-	-	-
98	A/135/21	19	29	M	-	-	-	-	HSK	-	-	-	-
99	**A/136/21**	22	23	M	-	-	-	-	AGT	-	-	-	677 C>T (rs1801133) heterozygous
100	A/137/21	21	22	F	-	-	-	-	-	-	-	-	-
101	A/138/21	20	23	M	-	-	-	-	-	-	-	-	-
102	A/139/21	22	28	M	-	-	-	-	-	-	-	-	-
103	A/140/21	23	20	M	-	-	-	-	-	-	-	-	-
104	A/141/21	25	37	M	-	-	-	-	-	-	-	-	-
105	A/142/21	28	33	F	-	-	-	-	-	-	-	-	-
106	**A/143/21**	30	24	M	A	-	-	-	-	-	-	-	677 C>T (rs1801133) heterozygous

107	A/146/21	24	20		-	-	-	-	PcK , AG	-	-	-	-
108	A/147/21	25	24	M	-	-	-	-	Rd	-	-	-	-
109	A/148/21	21	24	F	-	-	CCAM	Iah	AGT	-	-	SUA	-
110	A/150/21	26	32	M	-	-	-	-	-	-	-	-	-
111	A/151/21	32	31	M	-	-	-	-	GCK	-	-	-	-
112	A/152/21	20	26	M	-	-	-	-	-	-	-	-	-
113	A/153/21	20	27	F	-	-	-	Iah	-	Pf	-	-	-
114	**A/154/21**	29	24	F	-	-	-	-	PcK	-	-	-	677 C>T (rs1801133) heterozygous
115	A/155/21	22	28		-	-	-	-	AG	-	-	-	-
116	A/156/21	20	32	F	-	-	-	-	-	-	-	-	-
117	A/157/21	21	13	M	A, SB	-	-	-	-	-	-	-	-
118	A/158/21	23	28	F	-	-	-	-	-	-	-	-	-
119	A/159/21	24	24	M	H	-	-	-	-	-	-	-	-
120	A/160/21	23	34	M	-	-	-	-	-	-	-	-	-
121	A/161/21	25	32	F	-	-	-	DH	-	-	-	-	-
122	A/162/21	26	32	M	-	-	-	-	-	-	-	-	-
123	**A/163/21**	19	26	F	-	-	-	-	PcK	-	-	-	No targeted mutation
124	A/164/21	21	30	F	A,SB	-	-	-	-		CL, CP	SUA	-
125	A/165/21	19	32	F	-	-	-	-	-	-	-	-	-
126	A/166/21	26	32	F	-	-	-	-	-	-	-	-	-
127	A/167/21	22	27	M	-	-	-	Ia	-	-	-	SUA	-
128	A/168/21	21	24	F	-	-	-	-	-	-	-	-	-
129	A/169/21	20	24	F	A	-	-	-	-	-	-	-	-
130	A/170/21	20	24	F	-	-	-	-	-	-	-	-	-
131	A/172/21	28	30	M	-	-	-	-	-	-	-	-	-
132	A/173/21	24	13	M	-	-	-	-	-	-	-	-	-
133	A/174/21	26	29	M	-	-	-	O, Iah	-	-	-	SUA	-
134	A/175/21	26	22	F	-	-	-	-	-	-	-	-	-
135	A/176/21	28	28	F	-	-	-	-	-	-	-	-	-
136	A/178/21	27	22	F	-	-	-	-	-	-	-	-	-
137	A/179/21	25	19	F	-	-	-	-	-	-	-	-	-
138	A/180/21	26	26	F	-	-	-	-	-	-	-	-	-
139	A/181/21	22	28	F	-	-	-	-	-	CTEV	-	SUA	-
140	A/182/21	20	33	F	-	-	-	-	-	-	-	-	-
141	A/183/21	24	34	M	-	-	-	-	-	-	-	-	-
142	A/184/21	20	21	F	SB	-	-	-	-	-	-	SUA	-
143	A/185/21	24	28	M	-	-	-	-	-	-	-	-	-
144	A/186/21	23	16	F	-	-	-	O	-	-	-	-	-
145	A/187/21	21	24	M	-	-	-	-	-	-	-	-	-
146	A/188/21	24	20	F	-	-	-	TOF	-	-	-	SUA	-
147	**A/189/21**	21	22	M	-	-	HyL	Iah	PcK	-	-	-	-
148	A/190/21	26	20	M	-	-	-	DH	-	-	-	-	-
149	A/191/21	32	22	F	-	-	-	-	-	-	-	-	-
150	A/192/21	27	29	F	-	-	-	-	HSK	-	-	-	-
151	**A/193/21**	26	20	F	A	-	-	-	HyK, HyU	CTEV	SN	-	677 C>T (rs1801133) heterozygous
152	A/194/21	25	22	F	-	-	-	-	-	-	-	-	-
153	A/195/21	24	39	F	-	-	-	-	-	-	-	-	-
154	A/196/21	27	35	M	H	-	-	Iah	-	-	-	SUA	-
155	A/197/21	28	36	F	-	-	-	Iah, G	-	-	-	SUA	-
156	A/198/21	37	28	F	-	-	-	-	-	-	-	-	-
157	A/199/21	23	22	F	-	HyLHS	-	-	-	-	-	-	-
158	A/200/21	24	28	M	H	-	-	-	-	-	-	-	-
159	A/201/21	23	22	F	-	-	-	-	-	-	-	-	-
160	A/202/21	23	23	M	-	-	-	-	-	-	-	-	-