FETAL HYDROCEPHALUS: MATERNAL SERUM ALPHA FETO-PROTEIN LEVELS AND ASSOCIATED MALFORMATIONS

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By Terrence Paul Szajkowski

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree of

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FOR MY PARENTS
AND FOR KIM

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ABSTRACT

Fetal hydrocephalus is a condition whereby an unusually large amount of cerebrospinal fluid (CSF) accumulates in the fetal brain, most often in the cerebral ventricles. Counselling a patient after the ultrasonographic diagnosis of fetal hydrocephalus is difficult due to the heterogeneous nature of the condition and the unclear prognosis. The present study focuses on the analysis of maternal serum alpha-fetoprotein (MSAFP) levels and patterns of additional malformations in cases of fetal hydrocephalus. Several reports have suggested an association between elevated MSAFP and fetal hydrocephalus, but none of these studies have included data on a large, population-based sample. It is hoped that these studies will prove useful in the counselling and management of cases of fetal hydrocephalus.

One hundred and thirty-one cases were identified retrospectively for inclusion in the study. Seventy of these cases were used in the MSAFP analysis. Four independent-sample T-tests were performed. The first T-test compared the MSAFP levels in all cases of fetal hydrocephalus to the MSAFP levels in a control population of 91,541 women ($p = 0.029$). The second and third T-tests compared the MSAFP levels in the control population to those in the subsets of cases of complex fetal hydrocephalus ($p = 0.041$) and simple fetal hydrocephalus ($p = 0.203$), respectively. The fourth T-test compared the MSAFP levels in the subsets of simple and complex cases ($p = 0.198$). The sensitivity of MSAFP testing as a predictor for fetal hydrocephalus was poor, with only 7 cases (10%) falling at or above the "elevated" cut-off. However, 6 of the elevated cases (86%) had additional malformations.

Numerical taxonomy, a classification technique used to classify a large heterogeneous group of individuals into potentially more meaningful homogeneous subgroups, identified three distinct subgroups among the 78 cases of complex fetal hydrocephalus. Cluster one, consisting of 42 individuals, was the largest and most heterogeneous of the subgroups. Cluster two, comprised of 19 cases, was associated with central nervous system malformations. Cluster three, which included 17 individuals, was characterized by multiple malformations, especially those involving the craniofacial and musculoskeletal systems. Discriminant analysis correctly classified 94.9% of the cases when predicting cluster membership for the 78 cases of fetal hydrocephalus subjected to numerical taxonomy.

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1. INTRODUCTION

1.1 Hydrocephalus

Hydrocephalus is a condition whereby an unusually large amount of cerebrospinal fluid (CSF) accumulates in the brain, most often in the cerebral ventricles. The increase in fluid may be due to overproduction or lack of absorption, but is most often due to impediment of flow. The impeded flow prevents pulsatile pressure waves in the CSF from being dampened and leads to dilatation of the ventricles (ventriculomegaly). This may also manifest itself via an increase in head circumference, though this expansion is not always obvious prenatally or neonatally. Fetal hydrocephalus is often also associated with thinning of the calvaria and reduction of the cerebral cortex and white matter [1].

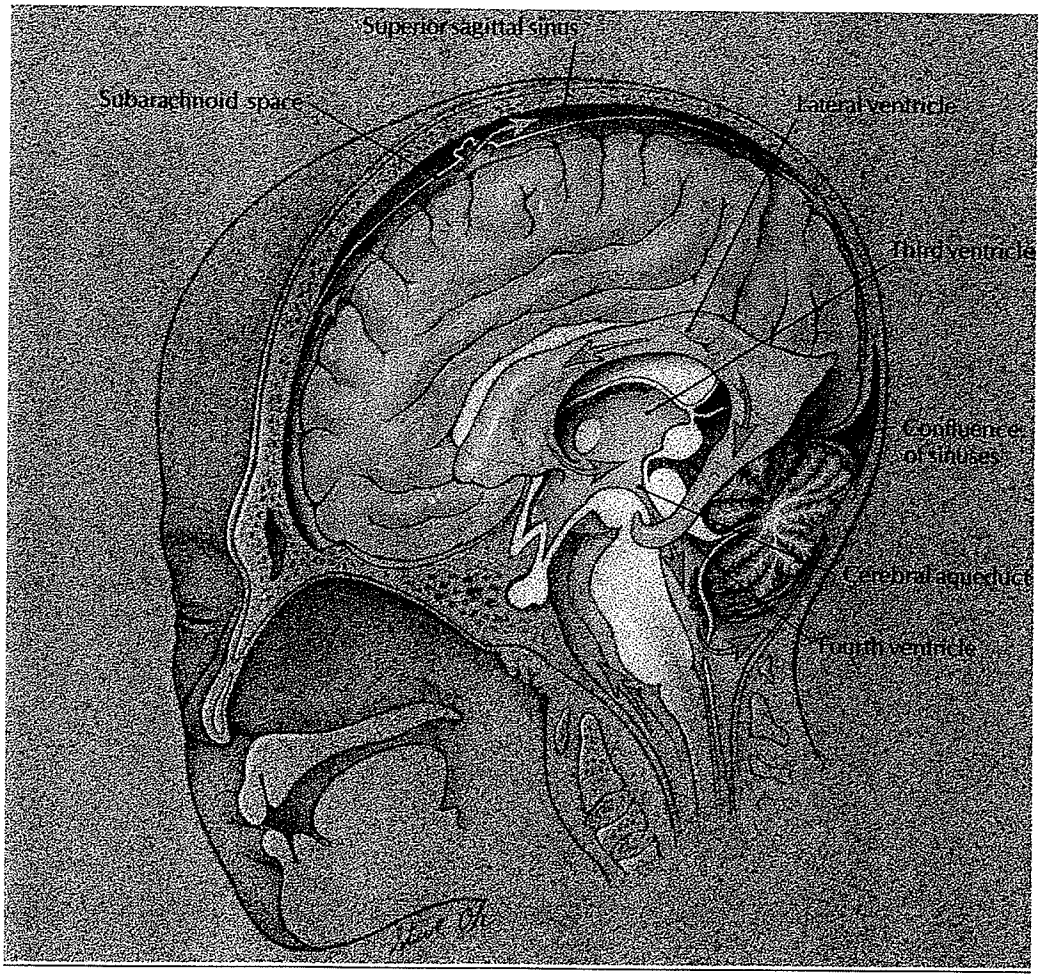
Counselling a patient after the diagnosis of fetal hydrocephalus is difficult due to the heterogeneous nature of the condition and the unclear prognosis. Information on biochemical maternal serum markers such as maternal serum alpha-fetoprotein (MSAFP) and early identification of any additional malformations may be useful in the counselling and management of cases of fetal hydrocephalus.

1.1.1 Cerebrospinal Fluid (CSF)

CSF is produced largely by the choroid plexus, a highly vascular structure found on the floor of the lateral ventricles and the roof of the 3rd and 4th ventricles. The path of the CSF starting from the lateral ventricles is as follows: from the lateral ventricles, CSF passes into the 3rd ventricle via the two foramina of Munro. It then travels into the 4th ventricle by way of the aqueduct of Sylvius. The fluid travels from the 4th ventricle into the subarachnoid space via the two lateral foramina of Luschka and the midline foramen

of Magendie. Eventually the fluid travels back to the site of reabsorption, the arachnoid villi of the sagittal and transverse sinuses, where it is reabsorbed into the blood. There is probably also reabsorption along cranial and spinal nerves into the lymphatic system. In healthy individuals, CSF is produced and reabsorbed at a rate of 0.3 ml/minute [2,3].

The physiological function of CSF in humans is threefold. First, it acts as a liquid cushion that pads and protects the brain. Second, it contains many nutrients that are essential for proper neurological function. Last, it provides a system for the elimination of wastes from the central nervous system [3]. The path of CSF in the ventricular system is shown in Figure 1.



From James [2].

Figure 1 Path of CSF in the Ventricular System

1.1.2 History of Hydrocephalus

The term hydrocephalus ("water on the brain") originated in ancient Greece. Hippocrates (460-377 B.C.) was the first to determine that the grossly enlarged head circumferences of some babies was due to the accumulation of a water-like substance in the head [4], though he believed the collection site of the fluid to be external to the brain [5]. It was more than 500 years later before anything new was learned about hydrocephalus. At that time, another Greek physician and philosopher named Claudius Galen (130-200 A.D.) undertook various experiments on animals where he managed to successfully dissect the ventricles of the brain and observed the release of a water-like fluid (CSF). Galen was the first to realize the importance of the choroid plexus in the production of CSF, though he was not fully aware of its role. Galen also described cases of hydrocephalus in his writings, but he, like Hippocrates, believed that the site of fluid collection was external to the brain and not in the ventricles themselves [6]. It was not until the work of Andreas Vesalius (1514-1563) that it was suggested that excess fluid within the ventricular system was the cause of the damage to the brain in hydrocephalus [5].

During the 17th and 18th centuries, a number of key discoveries regarding CSF flow dynamics and the pathophysiology of hydrocephalus were made. Thomas Willis (1612-1675) was the first to suggest that the choroid plexus is the site for CSF secretion and also that CSF was absorbed back into the blood stream via the venous system. Around the same time, Franciscus Sylvius (1614-1672) described the aqueduct that now bears his name. Giovanni Battista Morgagni (1682-1771) revealed that CSF freely communicated within the ventricles and the central and spinal arachnoid cavities and that impediment of passage of CSF through the aqueduct of Sylvius resulted in

hydrocephalus. Robert Whytt (1714-1766) documented the first clinical descriptions of hydrocephalus. In the mid-19th century, both the foramen of Magendie and the foramina of Luschka were described. Finally, in 1876, Key and Retzius fully described the complete path of CSF circulation as we understand it today [4,5].

1.1.3 Spectrum of Hydrocephalus

Due to the fact that hydrocephalus is a condition that may occur due to a variety of causes, no single ideal classification system exists. Hydrocephalus may be classified according to many different factors including time of onset, site of obstruction, fluid collection site, associated malformations, and others (Table 1) [7].

Hydrocephalus is a condition that may develop at any time during life. Prenatal (fetal) and neonatal hydrocephalus are collectively referred to as congenital hydrocephalus. Congenital ("at birth") hydrocephalus is often due to intrauterine infection or genetic causes, but may also be due to vascular disruptions or tumors if they occur in the prenatal or neonatal period. Infantile and adult onset hydrocephalus are most often acquired and are often due to haemorrhage, infection, neoplasms, or trauma [2].

Hydrocephalus is also often classified according CSF flow dynamics, which can be defined by the site of CSF obstruction or fluid collection. In communicating (or external or subarachnoid) hydrocephalus, an obstruction occurs in the basal cisterns that prevent CSF from being reabsorbed by the arachnoid granulations. This condition is

Table 1 Spectrum of Hydrocephalus

Specific factors	Types of Hydrocephalus
Time of Onset	Fetal Hydrocephalus
	Neonatal Hydrocephalus
	Infantile Hydrocephalus
	Adult Hydrocephalus
	Congenital Hydrocephalus
	Acquired Hydrocephalus
Obstruction Site	Communicating Hydrocephalus
	Noncommunicating Hydrocephalus
Fluid Collection Site	Internal or Intraventricular Hydrocephalus
	External or Subarachnoid Hydrocephalus
Associated Malformations	Simple Hydrocephalus
	Complex Hydrocephalus

Adapted from Mori [7].

most often an acquired process due to infection or subarachnoid hemorrhage. The subsequent inflammatory response that occurs results in scarring that prevents flow of CSF in the basal cisterns. In noncommunicating (or internal or intraventricular) hydrocephalus, CSF flow is inhibited in the ventricles of the brain, most often due to stenosis of the aqueduct of Sylvius. Hydrocephalus associated with other malformations is referred to as complex hydrocephalus [2,3,8].

1.1.4 Birth Prevalence

The birth prevalence of congenital hydrocephalus reported in the literature varies due to several factors. First, as for many other congenital anomalies, geographical differences in incidence of hydrocephalus exist. Second, prevalence will differ depending on whether total births (live born and stillborn) are considered versus just live births. The former situation is desirable as it gives a more accurate prevalence, as does the inclusion of prenatally diagnosed and terminated cases. Last, the exact definition of hydrocephalus used is also important. Although hydrocephalus and ventriculomegaly are often used synonymously, the two terms represent slightly different conditions. Hydrocephalus most often refers to an increase of CSF in the ventricular system associated with an increase in intracranial pressure, while ventriculomegaly is commonly defined as a simple enlargement of the ventricular system. In many cases ventriculomegaly will lead to hydrocephalus, but it may also resolve spontaneously. Instances where ventriculomegaly does not develop into full-blown hydrocephalus may be due to such causes as a temporary block in the CSF pathway or CSF production temporarily outpacing CSF pathway development [9].

Due to the above factors, the birth prevalence of congenital hydrocephalus reported in the literature varies considerably, with values ranging from 0.3-4.0 per 1000 total births, with the average prevalence being approximately 1.0 per 1000 total births [2,3,8,10-17]. There are more males than females affected due to the X-linked inheritance of some types of hydrocephalus.

1.1.5 Etiology

Congenital hydrocephalus can be a part of many different conditions, both syndromic and non-syndromic. Syndromic forms of congenital hydrocephalus include cytogenetic abnormalities, Mendelian conditions, and also some vascular disruptions. Trisomy 13, trisomy 18, trisomy 9p and triploidy are the most common cytogenetic abnormalities that may cause hydrocephalus. Conditions such as Walker-Warburg syndrome, Meckel syndrome and some types of VACTERL-Hydrocephalus syndrome are inherited in a Mendelian fashion and include hydrocephalus in their spectrum of anomalies. Conditions caused by vascular disruptions such as porencephaly and hydranencephaly may also be associated with congenital hydrocephalus [8].

Non-syndromic forms of congenital hydrocephalus are many and varied. Hydrocephalus is often associated with a neural tube defect. In fact, about 90% of children born with myelomeningocele also present with hydrocephalus. This is most often due to an obstruction of CSF flow at the base of the brain caused by a Chiari II malformation [3].

Isolated congenital hydrocephalus can also be inherited in a variety of fashions. The most common cause of familial isolated congenital hydrocephalus is X-linked

hydrocephalus associated with stenosis of the aqueduct of Sylvius. It has been estimated that approximately 2% of all cases of isolated congenital hydrocephalus are X-linked [18]. Mental retardation and adducted thumbs are commonly associated with this type of hydrocephalus, which is caused by a mutation in the gene for neural cell adhesion molecule L1 (L1CAM) [19], located at chromosome Xq28. Though much more rare than the X-linked form, both autosomal recessive [20,21] and autosomal dominant [13] forms of hydrocephalus associated with aqueductal stenosis have been proposed.

Other non-syndromic types of congenital hydrocephalus include those associated with a central nervous system malformation such as Dandy-Walker malformation or holoprosencephaly and also communicating hydrocephalus due to intraventricular subarachnoid haemorrhage, which is most often due to preterm delivery [8].

1.1.6 Recurrence Risks

The recurrence risk for congenital hydrocephalus depends on the etiology of the condition (Table 2). In cases where the cause is not known, empirical recurrence risk figures range between 1%-4% [8,16].

Table 2 Recurrence Risks in Congenital Hydrocephalus

1. Non-syndromic forms	
a. neural tube defect	2%-3%
b. aqueductal stenosis	
- previous affected child male	4%
-previous affected child female	2%
- L1CAM spectrum (male fetus)[a]	50%
- L1CAM spectrum (female fetus)[a]	<5%[b]
c. as part of a CNS malformation	
- Arnold-Chiari malformation	10%
- Dandy-Walker malformation	10%
- holoprosencephaly	0%[c]-50%
d. communicating	<3%
2. Syndromic forms	
a. cytogenetic abnormalities	
- aneuploidy	1%
b. Mendelian inheritance	
- autosomal recessive inheritance	25%
- autosomal dominant	0%[b]-50%
- X-linked	0%[b]-25%
3. Unknown	1%-4%

[a] when mother is a proven carrier
[b] female carrier has <5% chance of developing clinical features
[c] 0% in the case of a de novo mutation in the child

Adapted from Schrander-Stumpel and Fryns [8].

1.1.7 Hydrocephalus and Associated Malformations

A search of the London Dysmorphology Database [22] using various keywords ("aqueductal stenosis", "Arnold-Chiari malformation", Dandy-Walker malformation" and "non-specific hydrocephalus") yielded over 350 conditions associated with hydrocephalus. These conditions can be broken down into two groups: those involving hydrocephalus and other central nervous system (CNS) malformations and those involving hydrocephalus and non-CNS malformations.

1.1.7.1 Hydrocephalus and other CNS malformations

One condition that often includes hydrocephalus among its spectrum of CNS anomalies is Dandy-Walker malformation. This malformation is characterized by complete or partial agenesis of the vermis, cystic dilation of the fourth ventricle and an enlarged posterior fossa [23]. Another condition involving hydrocephalus and other CNS malformations is Walker-Warburg syndrome, an autosomal recessive disease relating to brain and eye anomalies. Walker-Warburg syndrome is characterized by lissencephaly, cerebellar malformation, retinal malformation and congenital muscular dystrophy [24]. Other CNS anomalies frequently found in cases of fetal hydrocephalus include agenesis of the corpus callosum and holoprosencephaly [12].

1.1.7.2 Hydrocephalus and non-CNS malformations

There are several conditions that may involve hydrocephalus in addition to non-CNS malformations. The classic triad of renal dysplasia, occipital cephalocele and postaxial polydactyly is the hallmark of Meckel-Gruber syndrome [25]. However, other

CNS malformations including hydrocephalus have also been reported in several cases [26]. Another disease called Hydrolethalus syndrome consists of hydrocephalus, polydactyly, micrognathia, midcranial malformations, visceral abnormalities and perinatal lethality [27].

The VACTERL association is a non-random grouping of vertebral (V), anal (A), cardiovascular (C), tracheo-esophageal (TE), radial and renal (R) and limb (L) anomalies first described by Quan and Smith [28]. In 1983, Sujansky and Leonard suggested that the VACTERL association in conjunction with hydrocephalus might represent a new autosomal recessive syndrome [29]. Since then, VACTERL-H has been shown to be both a causally and phenotypically heterogeneous disease with variable outcome. In addition to autosomal recessive inheritance, X-linked transmission of the disorder has also been proposed [30]. The spectrum of anomalies seen in VACTERL-H has been expanded to include microphthalmia, aberrant lung lobation and branchial arch defects [31,32]. Pregnancies involving VACTERL-H generally have very poor outcomes [31], although this is not always the case [33].

Many of the anomalies found in the spectrum of VACTERL-H overlap with those of Fanconi anaemia (FA), an autosomal recessive disease characterized by congenital anomalies, defective haematopoiesis [34] and increased chromosome breakage in cells exposed to the DNA cross-linking agent mitomycin C [35]. However, in some cases cells from patients previously diagnosed as having VACTERL-H have also been shown to exhibit increased chromosome breakage when subjected to mitomycin C [35,36], thus raising the question of whether VACTERL-H may be a severe form of FA in some cases [37]. The absence of excess chromosome breakage in cells exposed to mitomycin C from other patients with VACTERL-H [38] and the excess chromosome breakage in a family with

apparent X-linked VACTERL-H [39] has done little to clarify the situation and underlines the heterogeneous nature of the disease.

1.1.8 Mortality and Morbidity

When considering the outcomes of cases of congenital hydrocephalus, it is important to make a distinction between cases diagnosed prenatally and those diagnosed after birth. Hydrocephalus diagnosed prenatally has a much worse prognosis than hydrocephalus diagnosed postnatally. For the purposes of prenatal counselling, only data concerning the outcomes of pregnancies of congenital hydrocephalus diagnosed in utero should be used.

The overall outcome of pregnancies with a hydrocephalic fetus is generally, but not universally, poor. A study by Holzgreve et al. looked at a series of 118 cases of prenatally diagnosed hydrocephalus. Of the 118 cases, there were 27 (23%) terminations of pregnancy, 1 (1%) spontaneous abortion, 9 (8%) stillbirths, 15 (13%) deaths within 24 hours, 16 (14%) deaths from 24 hours to 28 days, 5 (4%) deaths after 28 days, and 45 (38%) surviving live births, many of whom had long term sequelae. The sub-group of cases of hydrocephalus with associated malformations had a worse prognosis [40].

In general, the best prognosis is for fetuses with mild ventriculomegaly, no associated anomalies, and a normal karyotype [9]. However, cases of isolated fetal hydrocephalus are rare. A study by Chervenak et al. [6] found that, in a series of 53 cases of fetal hydrocephalus, only 9 (17%) cases were isolated, while 44 (83%) cases had associated anomalies in one or more of the neurological, cardiovascular, renal, gastrointestinal, skeletal, respiratory, and reproductive systems.

A separate study by Chervenak et al. [17] looked at the intellectual outcomes of a group of 37 infants that had been diagnosed with hydrocephalus in utero. Among the survivors, a low intelligence quotient (I.Q.) was highly correlated with the presence of additional anomalies, especially other abnormalities of the CNS. None of the infants with normal I.Q. had associated anomalies.

1.1.9 Diagnosis and Treatment

The most common method of diagnosing hydrocephalus in utero is by ultrasound, although magnetic resonance imaging is used in rare instances [41]. Hydrocephalus can be detected by ultrasound as early as the end of the first trimester but is more clearly visible between 20-24 weeks gestation [3]. The lateral ventricle/hemispheric width ratio is one of the most common measurements used to identify fetal hydrocephalus, although other measurements have been suggested [42]. In the case of hydrocephalus due to aqueductal stenosis, there is dilation of the lateral and 3rd ventricles with a normal sized 4th ventricle. In hydrocephalus due to Dandy-Walker syndrome, it is usually only the 4th ventricle that appears dilated. Communicating hydrocephalus presents with dilatation of all four ventricles.

If possible, initial attempts to correct hydrocephalus after birth should focus on treatment of the underlying cause of the disease, e.g., removal of a tumour. In instances where it is not possible to treat the underlying cause, CSF shunting is the most common procedure used to treat hydrocephalus postnatally. In utero shunting attempts have proven largely unsuccessful due to the high procedure-related mortality rate [43,44]. The basic function of the shunt is to redirect CSF from the site of the obstruction to another

location in the body where it can be reabsorbed. Three types of shunts are commonly used. The ventriculoperitoneal (VP) shunt is the most popular type as extra tubing can be stored in the peritoneal cavity to allow for future growth of the patient. The ventriculoatrial (VA) shunt is used as an alternative to the VP shunt in patients who cannot tolerate peritoneal surgery. The lumboperitoneal shunt (LP) may be used in cases of communicating hydrocephalus [2].

A more recently developed technique used to correct some cases of noncommunicating hydrocephalus is third ventriculostomy. In this procedure tiny perforations are made in the wall of the 3rd ventricle with the aid of an endoscope, allowing the escape of CSF from the site of the blockage.

1.2 Alpha-fetoprotein

Alpha-fetoprotein is a glycoprotein initially produced by the yolk sac, then by the fetal liver during pregnancy. Discovered in 1956, AFP is the second most abundant protein in mammalian embryonic serum. It is a 591 amino acid protein with a molecular weight of 70,000 kDa, located at 4q11-q13. Although the function of AFP has still not been confirmed, it has been postulated that it may play a role in transport due to its multiple binding ligands [45]. AFP is detectable by the fourth week of gestation in the human embryo [46], and reaches its highest concentration of 3 mg/ml at about fourteen weeks of gestation [47]. From then, the concentration of AFP in fetal blood decreases to almost negligible levels at term.

From the fetus, AFP is passed via fetal urine into the amniotic fluid where it is referred to as amniotic fluid alpha-fetoprotein (AFAFP). AFAFP can then pass into the

maternal circulation, where it is referred to as maternal serum alpha-fetoprotein (MSAFP); via transmembranous transport from the amniotic cavity, or by diffusion across the placenta. Normally, about 94% of MSAFP is derived by diffusion across the placenta, while 6% comes from transport from the amniotic cavity [48].

1.2.1 AFP in Prenatal Screening

The value of using AFP levels as a screening test was first recognized in Scotland in 1972, when it was noted that neural tube defects such as spina bifida and anencephaly were associated with elevated AFAFP levels [49]. It was postulated that the cause of the elevated AFAFP levels was leakage of AFP-containing CSF across the open neural tube defect. Several other causes of elevated AFAFP have been identified including ventral wall defects such as gastroschisis and omphalocele, where transudation of AFP into the amniotic fluid occurs across blood vessels protruding from the abdomen. Congenital nephrosis may also lead to elevated AFAFP due to fetal proteinuria, as does any malformation of the gastrointestinal tract that causes impaired reabsorption of AFP from the amniotic fluid, e.g. esophageal atresia [50]. In all of these instances, MSAFP may also become elevated due to diffusion of the AFP across the amnion. However, defects in the placenta can lead to elevated MSAFP without causing an elevation in AFAFP levels [51]. Table 3 lists conditions reported in association with elevated MSAFP. It should be noted that for many conditions listed in the table this association was based on one or a few case reports only. Some conditions such as trisomy 18 and Down syndrome have subsequently been shown to be associated with *low* MSAFP levels [49]. Conditions that are considered to have a strong association with elevated MSAFP are shown in bold.

Today, MSAFP testing is preferred over AFAFP testing as a screening tool in pregnancies for several reasons. It is a less invasive test than the amniocentesis needed to obtain AFAFP, requiring only a maternal blood sample. As well, the cost and time necessary to obtain a blood sample are much less than for amniocentesis.

Table 3 Conditions Reported In Association with Elevated MSAFP

Abruptio placenta
Acardia
AFP, familial elevation
Amniotic band disruption sequence
Anencephaly
Aplasia cutis congenital
Breus mole
Bronchial atresia, main stem
Chorioangioma, placenta
Chromosome XXX syndrome
Chromosome XYY syndrome
Congenital nephrosis
Cojoined twins
Cranioachischisis
Cystic adenomatoid
Cytomegalovirus
Dandy-Waker Syndrome
Diastematomyelia
Disorganization mutation
Down Syndrome
Duplication 18p
Ectopic pregnancy
Encephalocele
Epidermis bullosa simplex
Exstrophy of the cloaca
Fetal alcohol syndrome
Fetal demise
Fetal distress
Fetus papyraceous
Fryns syndrome
Galloway-Mowat syndrome
Gastroschisis
Glutaricaciduria, type IIA
Hemangioma, umbilical cord
Herpes type I (necrosis fetal liver)
Hydrocephalus
Hydrops fetalis, idiopathic
Iniencephaly
Intrauterine growth retardation
Isochromosome 20q mosaicism
Klinefelter syndrome
Kniest dysplasia
Larsen syndrome
Limb-body wall complex
Lowe oculocerebrorenal syndrome
Meckel syndrome
Microcephaly
Monosomy 1q
Multiple gestation
Omphalocele
Parvo-virus
Polycystic kidney disease
Potter Sequence
Pulmonary valve atresia
Simpson-Golabi-Behmel syndrome
Smith-Lemli-Opitz syndrome
Spina bifida
Teratoma, sacrococcygeal
Tetralogy of Fallot
Tetraploidy mosaicism of placenta
Translocation chromosome Y;14
Transposition of the great vessels
Triploidy
Triploidy, XXYY aneuploidy
Trisomy 2, fetal mosaicism
Trisomy 13 syndrome
Trisomy 16
Trisomy 18 syndrome
Trisomy 20, mosaicism
Turner syndrome
Turner syndrome, isochrom. Xp
Umbilical cord abnormalities
Urethral obstruction malf. sequence
Varicella
Ventricular septal defect
XXYY syndrome
X;Y translocation
45,X male
46,XX/47,XXY mosaicism

Adapted from Weaver [52].

1.2.2 MSAFP Screening in Manitoba

After a two year pilot project, the Manitoba Maternal Serum AFP Screening Program became a formal provincial program funded by the Manitoba Health Services Commission on April 1, 1985. A programme coordinator runs the program and the overseeing committee includes a clinical director, research director, head of the fetal assessment unit, fetal assessment nurse, prenatal genetic counsellor, chief technologist and laboratory manager. The program operates with the cooperation of the Fetal Assessment Unit at the Health Sciences Centre, where most women are offered follow-up if necessary, and Cadham Provincial Laboratory, where the samples are sent for testing [53].

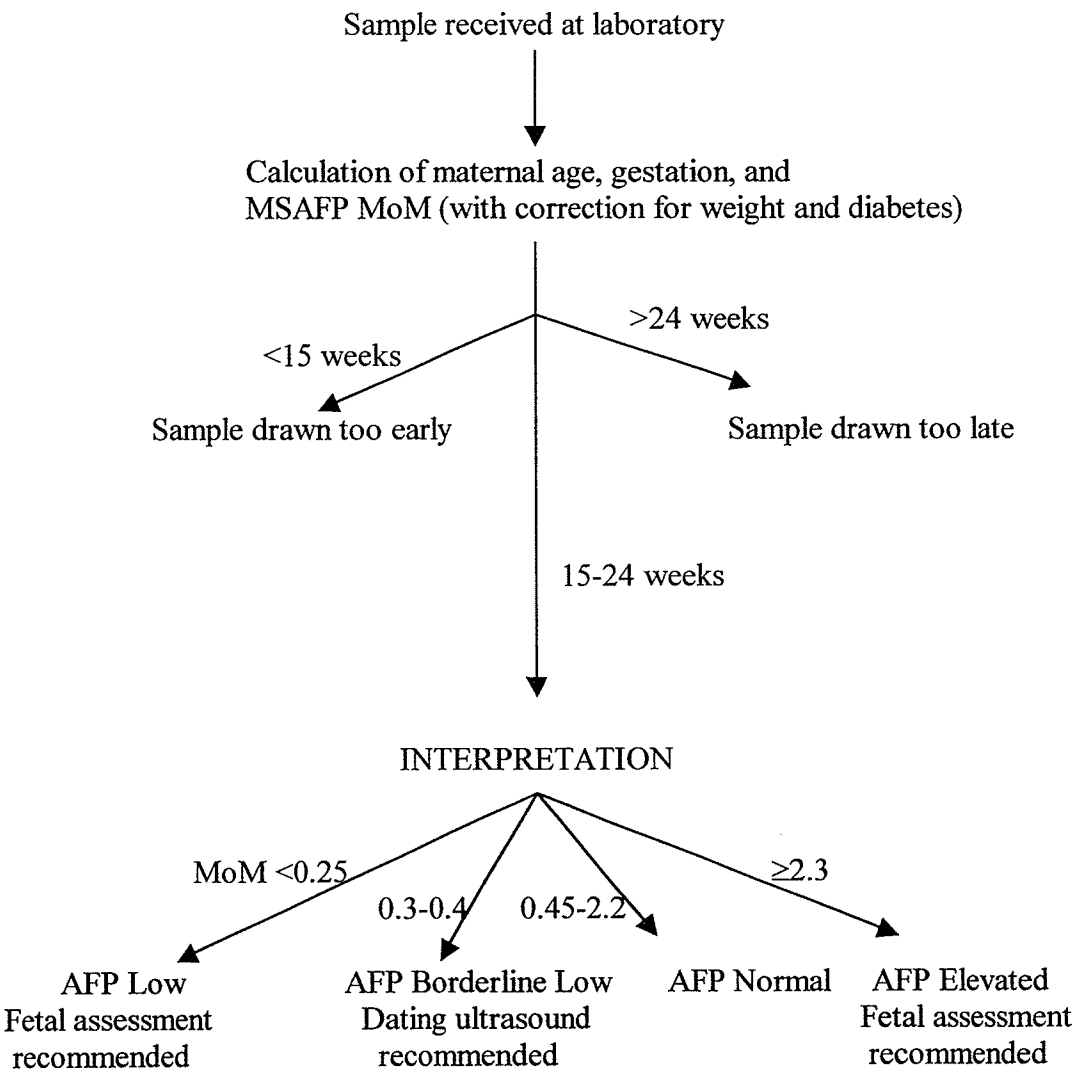
The current program protocol is shown in Figure 2. Samples are ordered by the family physician or obstetrician and sent to Cadham Provincial Laboratory for biochemical testing. The best time for screening is between 16-18 weeks of gestation, but it is possible to interpret MSAFP results anywhere from 15-24 weeks of gestation. Gestation will be determined either by ultrasound or based on the date of the last menstrual period. As maternal weight or diabetes can influence the interpretation of the screen, physicians are asked to supply information on these variables on the requisition form. Heavier women tend to have lower MSAFP concentrations while diabetic women have MSAFP levels that are approximately 20% lower than non-diabetic women. A multiple of the median (MoM) value is calculated for each sample by dividing the measured AFP (in micrograms per litre) by the median AFP for gestational age and then multiplying by the correction factors for weight and diabetes status. A fetal assessment (ultrasound) is recommended for any patient with an MSAFP of $\geq$2.3 MoM. If the

elevation is found to be due to wrong dates or a multiple gestation, the MSAFP is reinterpreted. If the elevation is due to a fetal anomaly, appropriate counselling is given. If no explanation can be determined for the MSAFP elevation, follow-up assessments are scheduled at 20-22 weeks and 30-32 weeks gestation to monitor fetal development.

Two points should be stressed regarding the MSAFP screening procedure. First, even though the test is offered to all pregnant women in Manitoba, it is completely voluntary and women can decline testing if they choose. Second, the test is only a screening tool for potential problems in a pregnancy. It is not a diagnostic test. As such, an abnormal result does not necessarily mean there is a problem with the fetus. Approximately 4% of all pregnant women have elevated MSAFP levels. Of these, only about 4% will have a fetus with an abnormality [54].

In May of 1999, the program changed its name to the Manitoba Maternal Serum Screening Programme. In addition to AFP, human chorionic gonadotrophin and estriol are now also screened for in maternal serum. The combination of tests is known as the "triple test" and is most useful in identifying fetuses at risk for chromosomal disorders such as Down syndrome and trisomy 18. Results are now reported in two categories – those related to the triple test and those related to the MSAFP test alone [54].

Figure 2 MSAFP Screening Protocol in Manitoba



Adapted from Chodirker and Evans [53].

1.2.3 AFP and Fetal Hydrocephalus

Seppala and Unnerus first noted the possible association between elevated AFP levels and fetal hydrocephalus in 1974 [55]. They reported high AFAFP concentrations in four cases of fetal hydrocephalus seen at their clinic. However, in all four cases, the MSAFP values were within the normal range. They postulated that the increase in AFAFP levels was due to passage of AFP-containing CSF through the greatly thinned fetal skull. Since then there have been relatively few instances of fetal hydrocephalus being reported in association with either elevated AFAFP [47,56] or MSAFP [57,58], but none of these studies included data on large, population-based samples.

1.3 Numerical Taxonomy

Numerical taxonomy is a classification technique that uses mathematical and statistical procedures to classify a large heterogeneous group of individuals into potentially more meaningful homogeneous subgroups. Initially developed for use in physical anthropology in the early 1900s, its true potential as a classification technique was not fully realized until the 1950s when computerized technology became more widely available. Since that time numerical taxonomy has further developed in both methodology and application and is now used in such areas as geology, zoology, botany, agriculture, psychology and the study of birth defects [59].

The most popular numerical taxonomy technique, referred to as polythetic agglomerative, is based on grouping individuals together to form larger groups on the basis of several characteristics at once. Together, the characteristics will collectively

define the group. In this type of numerical taxonomy, the data will consist of a population of individuals represented by observations of several characteristics referred to as attributes. In the study of birth defects, for example, data are most often recorded in binary form, i.e., the malformation is either present or absent. Recording this information for each attribute under consideration for the entire study population will yield a data matrix.

The next step in numerical taxonomy, once the data matrix has been constructed, is to quantify the similarity (or dissimilarity) between each and every pair of individuals in the dataset. Various measures of similarity can be used. Most binary data measures of similarity will yield coefficients between 0 and 1, with 0 denoting that two individuals have no attributes in common and 1 signifying that the individuals share exactly the same set of attributes. After coefficients of similarity have been calculated, individuals are grouped together using a sorting strategy that attempts to place the individuals with the greatest overall similarity together into the same subgroup or "cluster". A graphical representation of the clusters is called a dendrogram.

The final step in numerical taxonomy is interpretation of the results. The clusters should be defined with respect to the key attributes present and an attempt to determine underlying biological relationships should be made. This step may involve some hypothesizing by the researcher as to the reason for the relationships [60].

1.4 Objectives And Hypotheses

The objectives of this study were as follows:

1. To ascertain a population of fetal hydrocephalus cases in which MSAFP levels had been determined.

2. To record the distribution of MSAFP levels in this population and compare it to the distribution in all screened pregnancies.

3. To determine the sensitivity of MSAFP as a predictor for fetal hydrocephalus.

4. To compare the distribution of MSAFP levels in both isolated hydrocephalus and in cases with additional malformations to the distribution in all screened pregnancies.

5. To compare MSAFP levels in isolated hydrocephalus with those in cases with additional malformations.

6. To identify, using numerical taxonomy techniques, patterns of malformations associated with fetal hydrocephalus.

There were three hypotheses tested in this study:

1. MSAFP levels in cases of fetal hydrocephalus will be significantly higher than MSAFP levels in the general population of screened pregnancies.

2. Elevations in MSAFP levels in fetal hydrocephalus may be due to other malformations.

3. Of those cases of fetal hydrocephalus with associated malformations, distinct subgroups exist.

2. METHODS

2.1 Study Design

The design chosen for this investigation was that of a retrospective, population based study, which attempted to achieve as complete ascertainment as possible. The cases included all fetuses identified with hydrocephalus in pregnancies where the estimated date of delivery was between January 1st, 1985 and December 31, 2001. There was no systematic attempt to include cases before January 1st, 1985 but a few cases identified by the Manitoba Maternal Serum Screening (MMSS) programme before this date were also included as they contained complete data. Given that MSAFP evaluation was the major theme of the study, the year 1985 was chosen as the start date for ascertainment because that was the year that the MMSS program (formerly the Manitoba Maternal Serum AFP Screening program) became a formal provincial program. Cases of hydrocephalus in association with a neural tube defect (NTD) such as spina bifida were excluded from the study, as this combination of defects is a separate clinical entity known to be associated with elevated MSAFP. As well, since the study was concerned with *fetal* hydrocephalus, cases of neonatal hydrocephalus due to intraventricular hemorrhage were also excluded. Data from cases of fetal hydrocephalus found in multiple gestation pregnancies were included in the numerical taxonomy analysis, but excluded from the MSAFP analysis. This is because multiple gestation is known to be associated with elevated MSAFP. The control group used in the MSAFP analysis included all singleton pregnancies screened in Manitoba between 1990-2001 inclusively, in which the mother was not known to be diabetic. Mothers with insulin-dependent diabetes mellitus were

removed from the control group to allow easier comparison with other studies, many of which use different methods to control for the slower rise in MSAFP in women with this condition.

Ethics approval for the study was obtained from the University of Manitoba Research Ethics Board.

2.2 Ascertainment of Cases

From the outset of the study, it was realized that complete ascertainment of all cases of fetal hydrocephalus during the study period might not be possible, as no single registry for congenital anomalies currently exists in Manitoba. However, an attempt was made to ascertain as many cases of fetal hydrocephalus as possible. Multiple sources were utilized including the MMSS database, the Section of Genetics and Metabolism database and the Health Sciences Centre patient database.

The starting point in the search for cases was the Manitoba Maternal Serum Screening Database. Computerized records including patient names, dates of birth, draw dates and AFP levels exist for all patients of the screening program since December 1st, 1989. Pregnancy outcomes are also available for over 80% of these screened pregnancies. At the time the records were searched, the database was complete with respect to pregnancies screened up to December 31, 1998. Both maternal and neonatal outcomes were coded according to the International Classification of Diseases (ICD-9) coding scheme. The databases of maternal and infant outcomes were searched for all ICD codes that could pertain to congenital hydrocephalus. These included:

742.3 – Congenital hydrocephalus

742.9 – Unspecified anomaly of brain, spinal cord, and nervous system

771.2 – Congenital toxoplamosis

655.0 – Central nervous system malformation in the fetus

763.1 – Other malpresentation, malposition, and disproportion during labour or delivery.

All MMSS charts obtained from the ICD code search were reviewed to confirm the diagnosis of fetal hydrocephalus and cases that met the inclusion criteria (fetal hydrocephalus not associated with a NTD, date of delivery between 1/1/85 and 31/12/2001) were included in the study.

The next phase of the case ascertainment was carried out using the database from the Section of Genetics and Metabolism at the Health Sciences Centre, a source that would be especially useful for those cases where the mother had not undergone maternal serum screening. The Section keeps a computerized database of all patients seen and/or specimens referred for genetic analysis. The database was queried for all patients seen with a diagnosis of fetal hydrocephalus (using related key words) and these charts were manually reviewed.

A third source of case ascertainment was the Health Science Centre hospital charts. The hospital database was searched for any patients that had either ICD-9 code 742.3 (congenital hydrocephalus) or 655.0 (central nervous system malformation in the fetus) since 1982, the year the pilot program for MSAFP screening began in Manitoba. Lastly, summary reports from autopsies performed at the Health Science Centre on fetuses or neonates with hydrocephalus were reviewed, not to ascertain new cases, but to identify additional anomalies that were not found in any of the other chart reviews.

2.3 Data Collection

Data were collected on several variables for each case (Figure 3). Data collected focused on such information as demographic variables, maternal variables, serum screening data, and pregnancy outcome data.

Figure 3 Datasheet Used to Collect Information on Cases

Kindred # :
MMSS File # :
MHSC# :

Maternal weight:
Maternal D.O.B.:
Maternal age
Pregnancy history:

MSAFP (MoM):
Gestational age at screening:
Date sample taken:
MSAFP subsequent samples:
AFAFP:
BCHG/Estriol
Fetal karyotype:

Pregnancy outcome:
Sex:
Birth weight:
Gestational age at delivery:
Twin pregnancy:

Cause of hydrocephalus:

Associated malformations:

Syndrome:

Other:

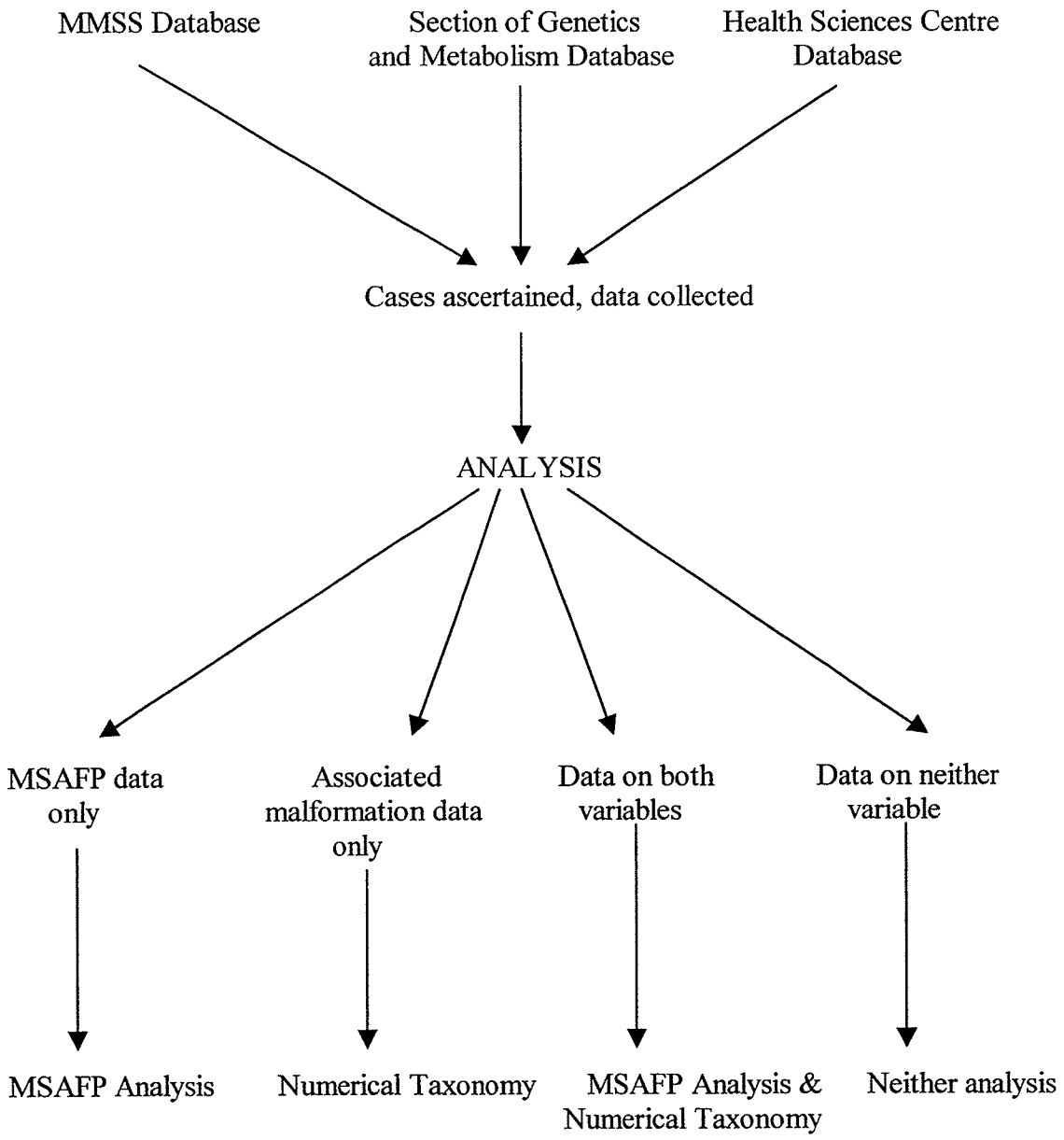
Kindred number (the Genetics clinic I.D. number), MMSS number, and MHSC number were identifying variables recorded so that it would be possible to revisit case files in the future should additional information be required. It was necessary to include all three I.D. numbers in the datasheet due to the fact that the various databases utilized different identification number schemes. In addition, each MMSS number is unique to a specific pregnancy while the kindred number and the MHSC number, as they apply to a family, might represent more than one case. Inclusion of these I.D. numbers helped to avoid duplication of records on the same case.

Maternal variables recorded included weight in kilograms (necessary for accurate calculation of MSAFP levels), date of birth, maternal age at birth of hydrocephalic child and pregnancy history. Maternal serum screening data documented included MSAFP level (in MoM) of the first appropriate sample drawn (i.e. at or after 15 weeks gestation), the date the sample was taken, gestational age in weeks, MSAFP level in subsequent samples, AFAFP level, beta-human chorionic gonadotrophin and estriol levels, if available, and fetal karyotype, if available. Pregnancy outcome data included pregnancy outcome (e.g. live birth, stillbirth, spontaneous abortion or therapeutic abortion), sex, birth weight, gestational age at delivery in weeks, twin pregnancy (yes or no), cause of hydrocephalus (if known) and any associated malformations. If a syndrome was identified in the fetus or neonate, this was also recorded. Lastly, any other notes made, such as the primary source for each case, were recorded in the field named "Other".

For the purposes of the study, the most important data collected were those concerned with the MSAFP levels and the associated malformations. Cases in which these variables were recorded were used in the MSAFP analysis, the numerical taxonomy

analysis or both. Figure 4 is a schematic showing how cases were ascertained and in which analysis they were used.

Figure 4 Schematic of Study Protocol



2.4 MSAFP Analysis

The SPSS 11.5 [61]Independent-Samples T-test (also known as the two sample t-test) was used to compare the mean MSAFP values of all the cases of fetal hydrocephalus and the control group. Mean MSAFP levels for both the subgroup of cases of complex fetal hydrocephalus and the subgroup of cases of simple fetal hydrocephalus were also compared to the control group. In addition, mean MSAFP levels in complex and simple cases were compared. MSAFP levels are reported in multiples of the median (MoM) so values can theoretically range from zero upward, with the expected median being about 1.0 MoM. Because of this, distributions derived from MSAFP data are characteristically skewed to the right. To normalize the distributions and allow use of the parametric T-test, the log of the MoM values of all MSAFP data was used in the analysis. The Levene test was used to test the assumption of homogeneity of variances between the case and control groups [62]. A significant result ($p \leq 0.05$) suggests that equal variances cannot be assumed when performing the Independent–Samples T-test. In this case, the T-test computation includes a correction for the lack of homogeneity of variance. T-test statistic values were evaluated for significance using a one-tailed test as the hypotheses related specifically to elevated MSAFP.

2.5 Sensitivity of MSAFP Screening

The sensitivity of MSAFP screening as a predictor for fetal hydrocephalus was determined at several "cut-off" points including 2.3 MoM, the level at which an MSAFP result is currently considered elevated in Manitoba. Other MSAFP cut-off levels chosen were 0.5, 0.8, 1.0, 1.5, 2.0 and 2.2 (MoM). The sensitivity was established by calculating

what proportion of screened cases of fetal hydrocephalus fell at or above each MSAFP level cut-off. In addition, the proportion of screened cases of fetal hydrocephalus that fell in a given MSAFP range and the proportion of all screened pregnancies in Manitoba from 1990-2001 that fell in the same MSAFP range were calculated. The MSAFP ranges reported included 0.0-0.49, 0.50-0.99, 1.00-1.49, 1.50-1.99, 2.00-2.49 and $\geq$ 2.50 (MoM).

2.6 Frequency of Mechanisms

If known, the exact cause of the fetal hydrocephalus was documented on the datasheet. This information was then used to determine the frequencies of the mechanisms of fetal hydrocephalus. Causes were coded from 1 to 5 as follows:

1. Aqueductal stenosis
2. Dandy-Walker malformation
3. Vascular disruption
4. Other
5. Unknown

Causes of fetal hydrocephalus included in the "Other" category were tumors, viruses, obstruction of the foramen of Munro, and Arnold-Chiari II malformation. A percentage was calculated for each category by dividing the number of cases in each category by the total number of cases overall. As well, a percentage was calculated for only those cases with a known cause by first excluding the "Unknown" category.

2.7 Numerical Taxonomy

All cases of complex hydrocephalus, i.e. cases of hydrocephalus in which there were additional malformations detected that were not considered to be a direct consequence of the hydrocephalus, were included in a numerical taxonomy analysis. A

coding sheet was developed based on the presence or absence of 41 different malformations (referred to as attributes), each of which was observed at least twice

Table 4 Associated Malformation Coding Sheet

Central Nervous System
1. Microcephaly
2. Agenesis/dysgenesis/hypoplasia corpus callosum
3. Holoprosencephaly
4. Abnormal cerebrum
5. Abnormal cerebellum
6. Other
7. Unspecified defect

Cardiovascular
8. Ventriculoseptal defect
9. Patent ductus arteriosus (not coded if <35 weeks gestation)
10. Hypoplastic left heart
11. Other
12. Unspecified defect

Respiratory
13. Lung lobation defects
14. Hypoplastic/dysplastic lungs

Gastrointestinal
15. Tracheoesophageal defects
16. Anal anomalies
17. Other

Urogenital
18. Renal a/dysplasia
19. Obstructive renal disease
20. Other urinary defect
21. Genital defects

Musculoskeletal
22. Cervicothoracic spine/rib anomalies
23. Lumbosacral spine anomalies
24. Limb deficiency defects
25. Digital defects
26. Positional limb defects
27. Rocker bottom feet
28. Diaphragmatic hernia
29. Other
30. Unspecified defect

Craniofacial
31. Cleft lip +/- palate
32. Cleft palate
33. Micrognathia
34. Low set ears
35. Other ear anomalies
36. Hypertelorism
37. Eye anomalies
38. Nose anomalies
39. Neck anomalies
40. Mouth anomalies
41. Skin defects

among the cases of complex hydrocephalus (Table 4). The information on each attribute was recorded in binary form, i.e., the anomaly was either present or absent. A polythetic agglomerative approach was used to analyze the data. In this method of numerical taxonomy, a similarity (or dissimilarity) coefficient is calculated for every combination of pairs of individuals in a data set. The type of similarity measure used in this analysis was Euclidian distance, which can only be applied to binary data and is a measure of dissimilarity between two individuals. Cases were then grouped in an agglomerative fashion using Wards sorting strategy. In this sorting technique, cases are fused together so that the operation causes the minimum increase of within-group sum of squared deviations from the mean [63]. The numerical taxonomy software used was Syn-Tax 2000 Hierarchical Clustering [64]. This program contains an "optimal cluster number" function that determines what number of clusters will explain the highest proportion of variance in the population. Lastly, each cluster was described and defined as to what key anomalies were present and possible underlying biological relationships were discussed.

2.8 Discriminant Analysis

After the completion of the numerical taxonomy and creation of the dendrogram, discriminant analysis was performed. This type of analysis utilizes pre-existing data (from the numerical taxonomy) to create a regression equation that maximally discriminates between the clusters. A stepwise procedure based on minimizing a statistical value called Wilks' lambda is used to select which attributes will be used as variables in the equation. For the purposes of this analysis, Wilks' lambda can be considered a measure of the usefulness of the regression equation in discriminating

between the clusters. Smaller Wilks' Lambda values signify greater power to discriminate. Variables are added to the regression equation in a step-by-step fashion based on their contribution to lowering the overall Wilks' Lambda value. In this way, the variables that most clearly help differentiate the clusters (i.e. have the most discriminating power) are added to the equation one at a time. Once a regression equation has been formulated it can be used to determine if each individual case was mathematically assigned to the same cluster it had been placed in by the numerical taxonomy. The equation can then also be used to help predict cluster membership in future cases [65].

3. RESULTS

3.1 Ascertainment of Cases

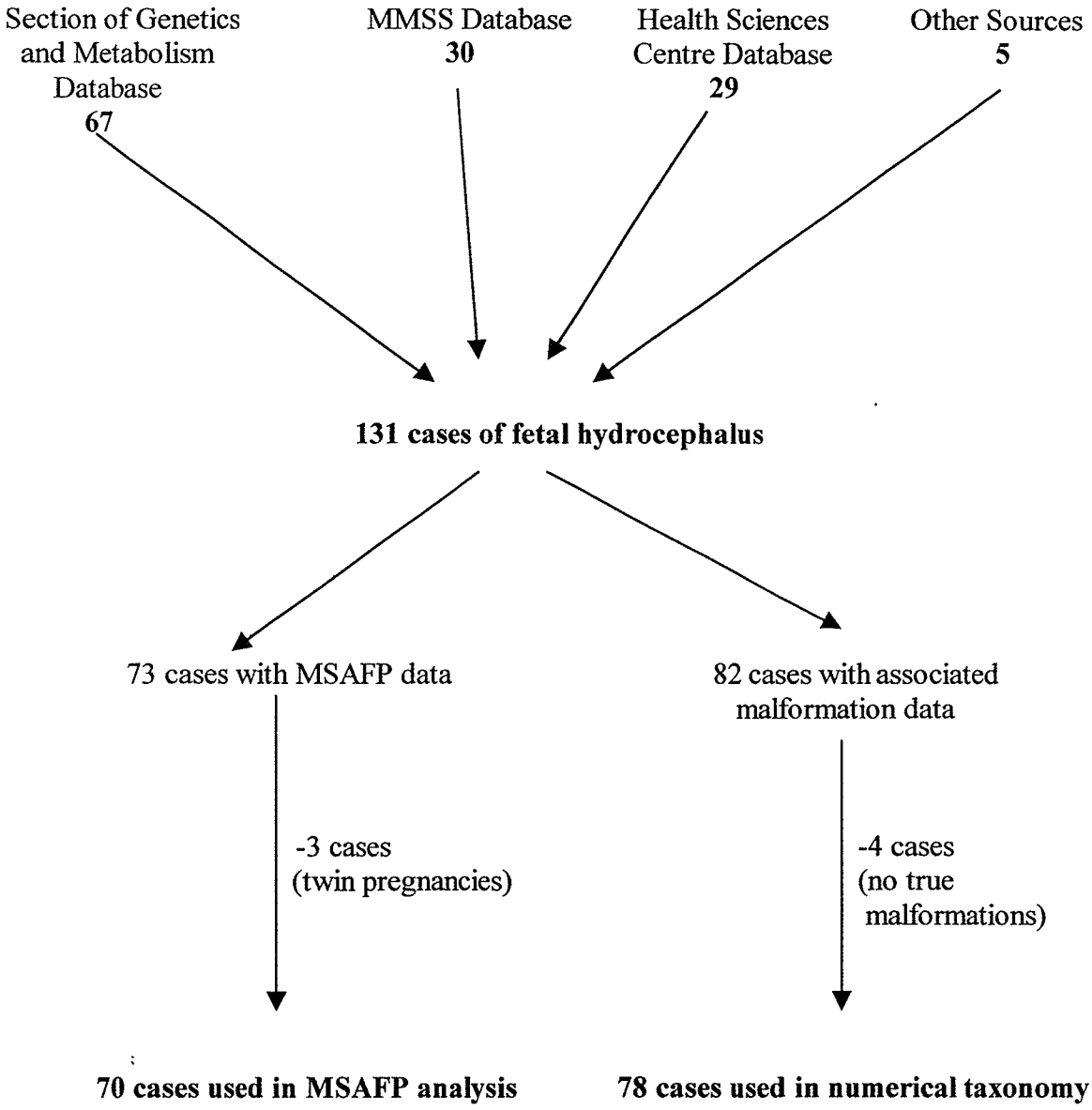
In total 131 cases of fetal hydrocephalus were ascertained. Of these, 67 were first identified through the Section of Clinical Genetics and Metabolism, 30 via the Manitoba Maternal Serum Screening database and 29 through Health Sciences Centre hospital charts. Five cases were found through other sources including autopsy reports and data previously presented at a conference by Karen MacDonald and Dr. Jane Evans [66]. Table 5 documents one such case to provide a summation of a typical case of fetal hydrocephalus with associated malformations.

Table 5 Summary of a Case of Fetal Hydrocephalus with Associated Malformations

History	Prenatal Diagnosis	Pregnancy Outcome
-Baby boy born at 35 weeks by cesarean section -Mother 27 y.o. G1P0 Canadian Aboriginal with well-controlled Type II diabetes mellitus -Father unrelated 28 y.o. -Planned pregnancy	-Ultrasound done at 6.5 weeks normal -MSAFP drawn at 17 weeks elevated at 2.6 MoM -Fetal assessment done at 20 weeks showed large head and abnormal intracranial structure -Amniocentesis results were 46, XY, AFAFP 2.6 MoM and Acetylcholinesterase gel negative	-Live birth, 2840g, Apgars 8^{1} 8^{5} -Following anomalies noted at birth: C7 hemivertebra, S3 R pedicle missing, T-E fistula with esophageal atresia, renal dysplasia left kidney, bilateral hydronephrosis and hydroureters, thin distally placed thumbs, micrognathia, micropthalmia -MRI revealed aqueductal stenosis. -Diagnosis: VACTERL-H -Baby had profound developmental delay, died at 3 y.o.

Of the 131 cases, 73 had MSAFP data and 82 had data on associated defects including 21 with recognized syndromes [VACTERL-H (9), Walker-Warburg syndrome (4), Trisomy 18 (1), Hydrolethalus syndrome (1), Meckel-Gruber syndrome (1), Fryns syndrome (1), Down syndrome (1), Ritscher-Schinzel syndrome (1), Fetal alcohol syndrome (1) and Amniotic band syndrome (1)]. Three cases with MSAFP data were excluded from the MSAFP analysis portion of the study due to twin pregnancies. Four cases were excluded from the numerical taxonomy portion of the study because their associated defects were not true malformations [neurosensory hearing loss (2), hyaline membrane disease (1) and hyperbilirubinemia (1)]. In total, 70 cases were included in the MSAFP analysis and 78 cases were subjected to numerical taxonomy. Approximately 25 cases had autopsy summary reports available. Twenty-two cases of fetal hydrocephalus were not able to be included in either analysis as they had no data on MSAFP levels or associated malformations. Figure 5 is a schematic summarization of the case ascertainment and exclusion.

Figure 5 Schematic for Case Ascertainment and Exclusion



3.2 MSAFP Analysis

Three different T-tests were performed using MSAFP data in this analysis (Table 6). The result of the Levene test for equality of variance was significant in three of the four comparisons, suggesting that equal variances could not be assumed when performing these tests. The first T-test compared the MSAFP values of all 70 cases of non-twin fetal hydrocephalus to the MSAFP values of the control population of all screened, singleton pregnancies in which the mother was non-diabetic from 1990-2001 (n=91,541).

Table 6 Group Statistics and T-test Results of MSAFP Analysis

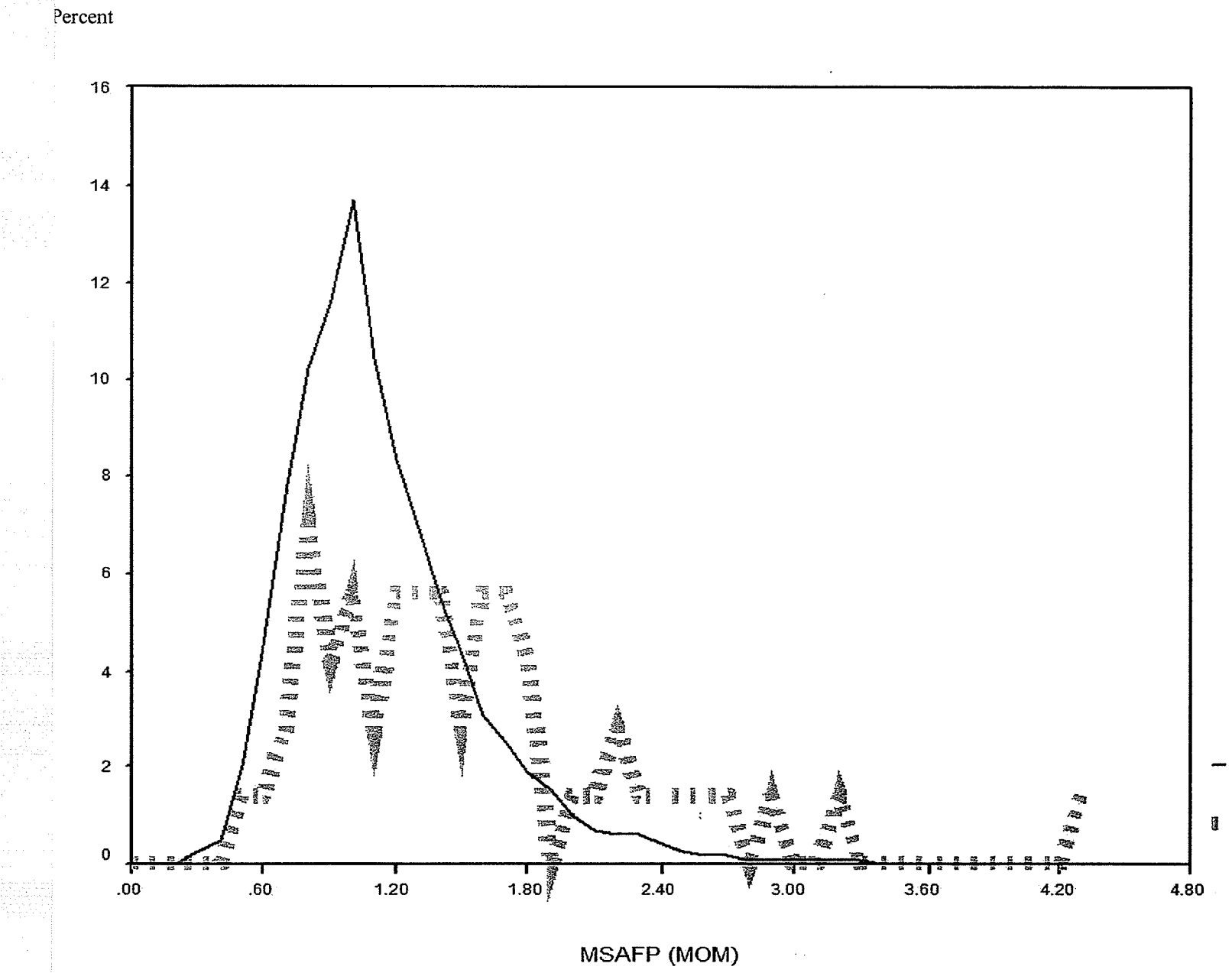
T-test	N	Mean MoM	Mean of Log values	Levene Test P-value	T-test P-value
All Cases	70	1.34	0.0727	0.000	**0.029**
Controls	91,541	1.14	0.0231		
Complex Cases	40	1.44	0.0910	0.003	**0.041**
Controls	91,541	1.14	0.0231		
Simple Cases	30	1.20	0.0484	0.493	**0.203**
Controls	91,541	1.14	0.0231		
Simple Cases	30	1.20	0.0484	0.037	**0.198**
Complex Cases	40	1.44	0.0910		

This test yielded a p-value of 0.029. Figure 6 shows the MSAFP distributions for the 70 cases of fetal hydrocephalus (both simple and complex cases included) and the control group.

The second T-test compared the MSAFP values of the complex cases of fetal hydrocephalus, i.e. those cases in which additional malformations were identified, to the control population. The p-value in this comparison was 0.041. The third T-test compared the MSAFP values of the simple cases (no additional malformations identified) to the control population and yielded a p-value of 0.203. The fourth T-test, comparing

the subsets of cases of complex and simple fetal hydrocephalus, resulted in a p-value of 0.196.

Figure 6 MSAFP Distributions in Cases and Controls



Red – MSAFP distribution of all screened pregnancies in MB from 1990-2001 (n=91,541)
Green – MSAFP distribution for cases of fetal hydrocephalus (n=70)

3.3 Sensitivity of MSAFP Screening for Fetal Hydrocephalus

Table 7 shows the sensitivity of MSAFP screening for fetal hydrocephalus at various cut-offs and the proportion of cases and controls that fall into each MSAFP range. The sensitivity of MSAFP screening at each cut-off is defined as the proportion of cases of fetal hydrocephalus that fall at or above that MSAFP level.

Table 7 Sensitivity of MSAFP Screening for Fetal Hydrocephalus and Distributions by Range

MSAFP Level *	Sensitivity of MSAFP screening at given cut-off (%)		MSAFP Range	Proportion of screened cases of fetal hydrocephalus in given range (%)	Proportion of all screened pregnancies (1990-2001) in given range (%)
0.5	97.1		0.00-0.49	2.9	1.5
0.8	83.8		0.50-0.99	37.1	40.6
1.0	60.0		1.00-1.49	25.7	39.7
1.5	34.3		1.50-1.99	18.6	13.1
2.0	15.7		2.00-2.49	7.1	3.3
2.2	12.9		≥2.50	8.6	1.8
2.3	**10.0**				

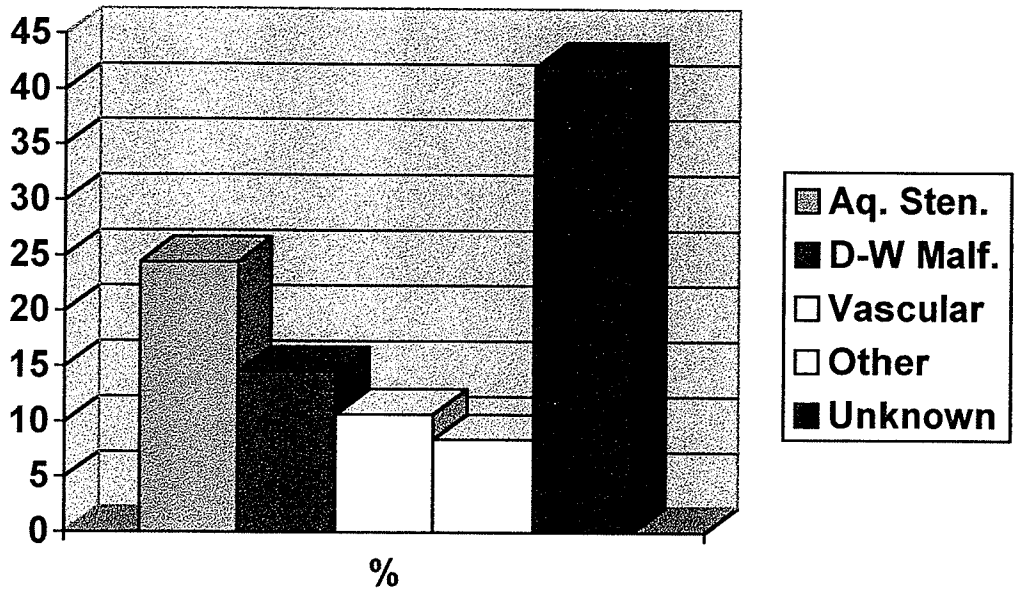
*MSAFP measured in multiples of the median. Values of 2.3 or greater are currently considered elevated in MB.

In Manitoba, MSAFP values of 2.3 MoM or greater are currently considered "elevated." Of the cases of fetal hydrocephalus that had MSAFP testing performed, 10.0% (7 cases) fell at or above this cut-off. In terms of the proportion of cases and controls in each range, a distinct pattern was seen. There were higher proportions of cases than controls in each of the upper ranges reported (1.50-1.99, 2.00-2.49 and ≥2.50 MoM). The lower ranges reported tended to have higher proportions of controls than cases in each range. The only exception to this was in the 0.00-0.49 MoM range, where there was a slightly higher proportion of cases than controls.

3.4 Frequency of Mechanisms of Fetal Hydrocephalus

Figure 7 shows the frequency of the different mechanisms in the 131 cases of fetal hydrocephalus. In 42.0 % of the cases the exact mechanism of hydrocephalus was unknown (n = 55). The most common known cause of fetal hydrocephalus was aqueductal stenosis (n = 32) followed by Dandy-Walker malformation (n = 19). The 14 cases of vascular disruptions causing fetal hydrocephalus included 6 cases of porencephaly, 7 cases of hydranencephaly, and 1 instance of twin-twin transfusion syndrome. The 11 individuals that fell into the "Other" category included 4 cases of tumors, 3 instances of obstruction of the foramen of Munro, 1 Arnold-Chiari malformation, 1 congenital Herpes simplex virus, 1 lobar holoprosencephaly, and 1 unknown intrauterine infection.

Figure 7 Frequency of Mechanisms of Fetal Hydrocephalus in All Cases (n=131)



3.5 Numerical Taxonomy

Seventy-eight (59.5%) of the original 131 cases of fetal hydrocephalus had associated malformations. The most common malformations are shown in Table 8. By system, associated malformations most often occurred in the central nervous system (48.7%), the craniofacial system (46.2%) and the musculoskeletal system (44.9%). Associated malformations occurred least often in the respiratory system. Only 12.8% of cases of complex fetal hydrocephalus had a malformation present in this system. Specific "attributes" that occurred in at least 10% of the cases included digital defects (23.1%), abnormal cerebrum (20.5 %), cervicothoracic spine/rib anomalies (15.4%), abnormal corpus callosum (15.4%), low set ears (14.1%), eye anomalies (14.1%), tracheoesophageal defects (12.8%), abnormal cerebellum (12.8%), micrognathia (11.5%), limb deficiency defects (10.3%) and renal a/dysplasia (10.3%).

Table 8 Selected Malformations in Cases of Complex Fetal Hydrocephalus (n=78)

Malformation	% of Cases	Number of Cases
Central nervous system	**48.7**	38
Abnormal corpus callosum	15.4	12
Abnormal cerebrum	20.5	16
Abnormal cerebellum	12.8	10
Cardiovascular system	**26.9**	21
Respiratory system	**12.8**	10
Gastrointestinal system	**17.9**	14
Tracheoesophageal defects	12.8	10
Urogenital system	**24.4**	19
Renal a/dysplasia	10.3	8
Musculoskeletal system	**44.9**	35
Cervicothoracic spine/rib anomalies	15.4	12
Limb deficiency defects	10.3	8
Digital defects	23.1	18
Craniofacial system	**46.2**	36
Micrognathia	11.5	9
Low set ears	14.1	11
Eye anomalies	14.1	11

The group of 78 cases of complex fetal hydrocephalus was subjected to numerical classification to determine if more homogeneous subgroups could be identified. The dendrogram depicting the results of the numerical taxonomy analysis is shown in Figure 9. There were three distinct clusters (subgroups). The number of cases in clusters one, two, and three were 42, 19, and 17, respectively. Pearson's Chi-Square test was performed on each attribute to determine if the pattern of distribution of the malformations among the clusters was statistically significant. Table 9 shows the results of the chi-square analysis. P-values for several attributes, including abnormal corpus callosum, abnormal cerebellum, renal a/dysplasia, cervicothoracic spine/rib anomalies, lumbosacral spine anomalies, limb deficiency defects, rocker bottom feet, cleft lip +/- palate, micrognathia, low set ears, eye anomalies and mouth anomalies were significant at the 0.01 level. This is strong evidence for a non-random distribution of these attributes among the clusters.

Figure 8 Dendogram Depicting the Results of the Numerical Taxonomy Analysis of Cases of Complex Fetal Hydrocephalus

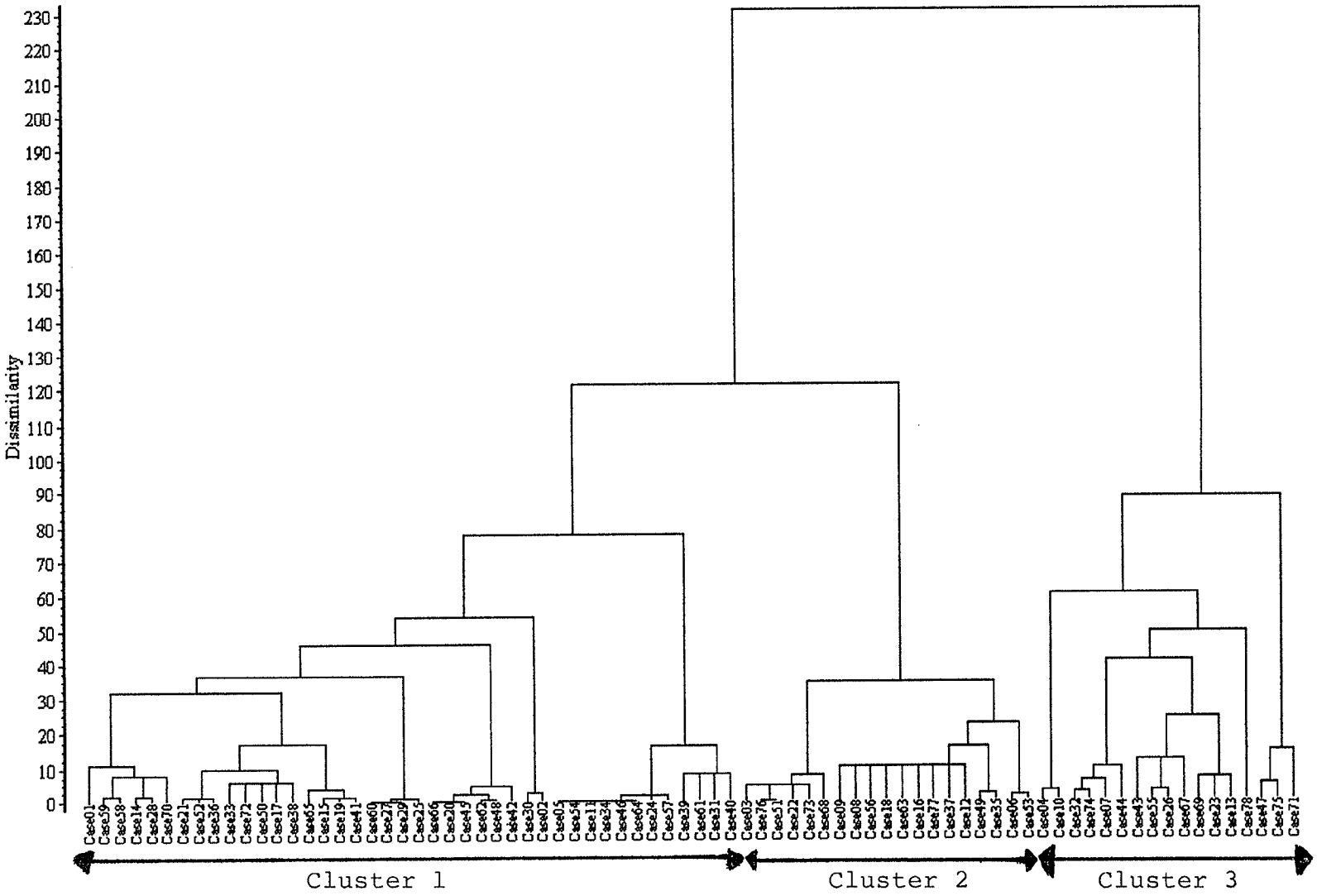


Table 9 Results of Pearson's Chi-Square Analysis for Each Attribute

Attribute	P-value
Central Nervous System	
1. Microcephaly	0.805
2. Agenesis/dysgenesis/hypoplasia corpus callosum	<0.001**
3. Holoprosencephaly	0.276
4. Abnormal cerebrum	0.389
5. Abnormal cerebellum	<0.001**
6. Other	0.041*
7. Unspecified defect	0.648
Cardiovascular	
8. Ventriculoseptal defect	0.028*
9. Patent ductus arteriosus	0.041*
10. Hypoplastic left heart	0.645
11. Other	0.157
12. Unspecified defect	0.164
Respiratory	
13. Lung lobation defects	0.341
14. Hypoplastic/dysplastic lungs	0.017*
Gastrointestinal	
15. Tracheoesophageal defects	0.911
16. Anal anomalies	0.028*
17. Other	0.534
Urogenital	
18. Renal a/dysplasia	0.009**
19. Obstructive renal disease	0.178
20. Other urinary defect	0.028*
21. Genital defects	0.060
Musculoskeletal	
22. Cervicothoracic spine/rib anomalies	<0.001**
23. Lumbosacral spine anomalies	<0.001**
24. Limb deficiency defects	0.009**
25. Digital defects	0.023*
26. Positional limb defects	0.497
27. Rocker bottom feet	0.004**
28. Diaphragmatic hernia	0.415
29. Other	0.028*
30. Unspecified defect	0.025*
Craniofacial	
31. Cleft lip +/- palate	0.004**
32. Cleft palate	0.028*
33. Micrognathia	<0.001**
34. Low set ears	0.001**
35. Other ear anomalies	0.823
36. Hypertelorism	0.289
37. Eye anomalies	<0.001**
38. Nose anomalies	0.093
39. Neck anomalies	0.137
40. Mouth anomalies	0.008**
41. Skin defects	0.137

*Significant at $p \leq 0.05$ level **Significant at $p \leq 0.01$ level

Cluster one, consisting of 42 individuals, was the largest and most heterogeneous of the subgroups. There did not seem to be a particular attribute or group of attributes that characterized the individuals in this group. However, it is interesting to note the lack of certain malformations. Despite the relatively large number of cases, there were no instances of abnormal cerebellum, abnormal corpus callosum, cervicothoracic or lumbosacral spine/rib anomalies, micrognathia, eye anomalies or mouth anomalies. The mean MSAFP level for those individuals who underwent screening (excluding the one case of a twin pregnancy) was 1.46 MoM in this subgroup.

Cluster two consisted of 19 individuals. A strong association with central nervous system malformations characterized this subgroup. In particular, cluster two had a high proportion of the abnormal corpus callosum (9 of the 12 instances of abnormal corpus callosum occurred in this cluster) and abnormal cerebellum (7/10) defects. This subgroup was also characterized by a strong association with eye anomalies (8/11). The mean MSAFP level in cluster two was 1.32 MoM.

Cluster three was the smallest subgroup with 17 cases. Most individuals in this cluster had multiple malformations. This subgroup was characterized by malformations of the musculoskeletal and craniofacial systems. In particular, cluster three had strong associations with cervicothoracic (12/12) and lumbosacral (6/7) spine/rib anomalies, limb deficiency defects (5/8), rocker bottom feet (3/3), cleft lip +/- palate (3/3), micrognathia (8/9), low set ears (7/11), and mouth anomalies (4/6). There was also a strong association with renal a/dysplasia (5/8). Cluster three had a mean MSAFP level of 1.54 MoM.

The Kruskal-Wallis non-parametric test was performed to compare the MSAFP values found in the three clusters. A p-value of 0.650 was obtained, suggesting no significant difference in the MSAFP levels among the subgroups.

The specific findings associated with each case of fetal hydrocephalus are shown in Appendix 1. The order of cases in the table corresponds to the order shown in the dendrogram. MSAFP values are also shown for those cases in which MSAFP testing was performed.

3.6 Discriminant Analysis

The stepwise statistics pertaining to the formulation of the regression equation and the distribution of each discriminating variable among the three clusters is shown in Table 10. For each step, the variable that minimized the overall Wilks' Lambda was entered. The multivariate significance at each step was $p<0.001$. The eight major distinguishing attributes were, in order of discriminating power, cervicothoracic spine/rib anomalies, micrognathia, abnormal corpus callosum, cleft lip +/- palate, musculoskeletal unspecified defects, eye anomalies, nose anomalies, and limb deficiency defects.

Table 10 Stepwise Statistics of Variables in the Regression Equation and Distribution Among the Clusters

Step	Variable Entered	Wilks' λ	Cluster 1 n (%)	Cluster 2 n (%)	Cluster 3 n (%)
1.	Cervicothoracic Spine/Rib Anomalies	0.348	0 (0)	0 (0)	12 (100)
2.	Micrognathia	0.211	0 (0)	1 (11.1)	8 (88.9)
3.	Abnormal Corpus Callosum	0.147	0 (0)	9 (75)	3 (25)
4.	Cleft Lip +/- Palate	0.111	0 (0)	0 (0)	3 (100)
5.	Musculoskeletal Unspecified Defects	0.086	0 (0)	0 (0)	2 (100)
6.	Eye Anomalies	0.068	0 (0)	8 (72.7)	3 (27.3)
7.	Nose Anomalies	0.056	1 (20)	1 (20)	3 (60)
8.	Limb Deficiency Defects	0.048	3 (37.5)	0 (0)	5 (62.5)

It is important to note that the eight most discriminating attributes are not necessarily the same as the attributes that had the smallest p-values in the chi-square analysis done in the numerical taxonomy portion of the study. This is because the emphasis in discriminant analysis is identifying the variables that will contribute most to the equation as a whole. For example, if two attributes show the exact same distribution among the subgroups, discriminant analysis will only select one for inclusion in the regression equation.

The regression equation derived from the discriminant analysis was reapplied to the original 78 cases of complex fetal hydrocephalus to see if their predicted cluster membership based on the 8 weighted variables (each variable has its own coefficient) would match their cluster assignment according to the numerical taxonomy. The result of the discriminant analysis classification is shown in Table 11. When this exercise was performed, 94.9% (74 of 78) of the cases were correctly classified. The four misclassified cases were all assigned to cluster two but predicted to be in cluster one.

It is unclear on initial examination why this misclassification took place. For example, one of the misclassified cases (I.D. #63) had only one attribute present – abnormal cerebellum. Seven of the ten instances of abnormal cerebellum are present in cluster two, yet the discriminant analysis predicted this case would fall into cluster one. However, "abnormal cerebellum" is not one of the eight most discriminating attributes used as variables in the regression equation. In fact, none of the attributes present in the misclassified cases appear as variables in the equation. In addition, of the attributes constituting the top six most discriminating variables, none are found in cluster one.

Apparently, the absence of any of these attributes in four cases led them to be misclassified into cluster one by the regression equation.

Table 11 Discriminant Analysis Classification Results

Case I.D. #	Assigned Cluster	Predicted Cluster		Case I.D. #	Assigned Cluster	Predicted Cluster
1	1	1		61	1	1
59	1	1		31	1	1
58	1	1		40	1	1
14	1	1		3	2	2
28	1	1		76	2	2
70	1	1		51	2	2
21	1	1		22	2	2
52	1	1		73	2	2
36	1	1		68	2	2
33	1	1		9	2	2
72	1	1		8	2	2
50	1	1		56	2	2
17	1	1		18	2	2
38	1	1		63	2	1*
65	1	1		16	2	1*
15	1	1		77	2	2
19	1	1		37	2	1*
41	1	1		12	2	2
60	1	1		49	2	1*
27	1	1		35	2	2
29	1	1		6	2	2
25	1	1		53	2	2
66	1	1		4	3	3
20	1	1		10	3	3
45	1	1		32	3	3
62	1	1		74	3	3
48	1	1		7	3	3
42	1	1		44	3	3
30	1	1		43	3	3
2	1	1		55	3	3
5	1	1		26	3	3
54	1	1		67	3	3
11	1	1		69	3	3
34	1	1		23	3	3
46	1	1		13	3	3
64	1	1		78	3	3
24	1	1		47	3	3
57	1	1		75	3	3
39	1	1		71	3	3

*Misclassified Case

4. DISCUSSION

4.1 Case Ascertainment

It was realized from the outset of the study that complete ascertainment of all cases of fetal hydrocephalus during the study period might not be possible, as no single registry for congenital anomalies currently exists in Manitoba. However, the hope was that by utilizing multiple sources of ascertainment as many cases of fetal hydrocephalus as possible would be identified. The three sources used to identify cases of fetal hydrocephalus in the study each had their own strengths and weaknesses. The Manitoba Maternal Serum Screening (MMSS) database was considered an obvious good choice for case ascertainment, as the comparison of MSAFP levels was an integral part of the study. However, as the test is voluntary, approximately 30-35% of pregnant women in the province decline testing [67]. The database from the Section of Genetics and Metabolism and the Health Sciences Centre hospital charts were also considered good choices for case ascertainment, especially for women who choose not to undergo MSAFP screening. Due to its often genetic or unknown etiology or its presence among multiple congenital anomalies, cases of fetal hydrocephalus are often referred to the Section of Genetics and Metabolism (the only such facility in the province). More deliveries take place at the Health Sciences Centre than in any other hospital in the province (especially in instances where a malformation that could potentially complicate the delivery has been identified prenatally). However, cases of fetal hydrocephalus that were not referred to the Section of Genetics and Metabolism or delivered at the Health Sciences Centre could potentially be missed.

In total, 131 cases were identified for inclusion in the study. Based on an average birth prevalence of 1/1,800 total births in Manitoba [31] and an approximate birth rate of 16,000 births/year in Manitoba during the 17-year study period (StatsCan), we would have expected to find about 151 cases of fetal hydrocephalus if complete ascertainment had been achieved. In any case, the method of case ascertainment used in the study resulted in a large, population-based sample of cases of fetal hydrocephalus. Also, although complete case ascertainment may not have been obtained, there was no indication of any biases in the ascertainment that may have affected the results.

4.2 MSAFP and Fetal Hydrocephalus

Since Seppala and Unnerus [55] first suggested a possible association between elevated AFP levels and fetal hydrocephalus in 1974, there has been a relative lack of information about the subject in the literature. Some of the instances in which AFP levels were reported in cases of fetal hydrocephalus are listed in Table 12. Most of these instances are case reports based on few patients. One of the strengths of the present study is that its focus is a large, population-based sample of cases of fetal hydrocephalus.

Seppala and Unnerus postulated that the increase in AFAFP levels they observed in four cases of fetal hydrocephalus might be due to passage of AFP-containing CSF across the greatly thinned fetal skull into the amniotic cavity [55]. However, in their paper, none of the patients had elevated MSAFP levels. One possible explanation for

Table 12 AFP Levels Reported in Cases of Fetal Hydrocephalus

YEAR/AUTHOR	NUMBER OF CASES	SOURCE OF AFP	AFP LEVELS	NOTES
1975, Seppala [47]	7	Amniotic fluid	5 elevated, 2 normal	
1978, Henrion et al. [68]	3	Amniotic fluid	1 elevated, 2 normal	AFAFP measured in third trimester.
1981, Klink et al. [56]	3	Amniotic fluid	3 elevated	
1981, Robertson et al. [69]	2	Amniotic fluid	2 normal	Two pregnancies in same family.
1983, Laurence et al. [70]	3	Amniotic fluid	3 normal	
1985, Augier et al. [71]	3	Maternal serum	2 elevated, 1 normal	Elevated cases $\geq 99^{th}$ percentile
1988, Burton [58]	6	Maternal serum	6 elevated	MoM ≥ 2.5
1990, Thomas and Blakemore [48]	1	Amniotic fluid Maternal serum	AFAFP normal MSAFP elevated	AFAFP 1.48 MoM MSAFP 14.65 MoM
1992, Sarandakou et al. [46]	2	Amniotic fluid	2 low	
1994, Goodburn et al [57]	7	Maternal Serum	7 elevated	5 ≥ 2.5 MoM; 2 ≥ 2.0 MoM
1996, Walker et al. [72]	1	Maternal Serum	1 normal	MSAFP 1.4 MoM Fetus had ring chrom. 6
1999, MacDonald et al. [66]	4	Maternal Serum	1 elevated, 3 above average	Hydrocephalus with VACTERL assoc. 2.6 MoM; 1.6 MoM; 1.6 MoM; 2.1 MoM

elevated MSAFP in cases of fetal hydrocephalus is that excess AFP in the amniotic fluid might pass into the maternal serum by diffusion across the fetal membranes.

Another hypothesis regarding elevations of MSAFP in fetal hydrocephalus is that they may actually be due to other undetected malformations present. In 1999 MacDonald et al. reported on a male infant in whom hydrocephalus was identified during a fetal assessment performed for elevated MSAFP [66] and who was found at birth to have VACTERL-H (case is summarized in Table 5). Three other similar cases were commented on as well. The authors hypothesized that MSAFP elevations in cases of

VACTERL-H may not be due to the fetal hydrocephalus but rather to other, harder to detect malformations. Esophageal atresia, which was present in all four cases but not identified until birth, was singled out as a possible cause of the MSAFP elevations because impaired swallowing might prevent reabsorption of AFAFP. A previous study by the same group had found an association between esophageal atresia and elevated MSAFP levels [73].

The first T-test performed in this study was done to test the hypothesis that MSAFP levels in cases of fetal hydrocephalus are significantly higher than MSAFP levels in the general population of screened pregnancies. Comparison of the 70 cases of fetal hydrocephalus with MSAFP data with the control population yielded a p-value significant at the 0.05 level ($p = 0.029$). This result confirmed the first hypothesis of the study.

The second and third T-tests performed in this study were done to test the hypothesis that any MSAFP elevation in cases of fetal hydrocephalus might be due to other malformations. The second T-test compared the MSAFP levels in the subset of cases of complex hydrocephalus with MSAFP levels of the control population. Again the p-value was significant at the 0.05 level ($p = 0.041$), suggesting that on a population level cases of complex fetal hydrocephalus do have significantly higher MSAFP levels than the general population of screened pregnancies. The third T-test comparing MSAFP values in the subset of cases of simple fetal hydrocephalus with those of the control population yielded a p-value of 0.203, suggesting that little or no difference exists in the MSAFP distributions of these two populations. Taken together, the results of the second and third T-tests confirmed the second hypothesis of the study. The fourth T-test, comparing the

complex and simple cases, did not show a significant difference ($p = 0.198$), potentially due to the small sample sizes.

These results are useful because they represent the first population-based analysis of MSAFP levels in fetal hydrocephalus. In accordance with several case reports suggesting an association between fetal hydrocephalus and elevated MSAFP, the present study found that this association exists on a population level. However, from a clinical standpoint, more useful information can be discerned from determining the sensitivity of MSAFP testing as a predictor for fetal hydrocephalus and examining the cases that fall at or above the "elevated" cut-off (currently 2.3 MoM in Manitoba).

In the present study, the sensitivity of MSAFP screening for fetal hydrocephalus was poor, with only 7 of 70 cases (10%) having values at or above 2.3 MoM. However, 6 of 7 (86%) of these cases had associated malformations. Two (29%) of the cases above the cut-off had obvious VACTERL-H with esophageal atresia among their spectrum of anomalies. These data strongly suggest that elevated (i.e. ≥ 2.3 MoM) MSAFP levels in pregnancies with fetal hydrocephalus do identify women whose infants are at significant risk to have additional malformations. This has implications for follow-up and genetic counselling. For instance, in addition to karyotyping, chromosome breakage studies may be indicated to test for the possible presence of VACTERL-H or Fanconi anemia in cases of fetal hydrocephalus. As well, detailed serial fetal assessments with ultrasound should be performed as some malformations such as esophageal atresia can be difficult to detect and other malformations such as hydronephrosis may only be evident later in gestation.

4.3 Numerical Taxonomy

Numerical taxonomy is a widely used classification technique whose purpose is to break down a large diverse group of objects into meaningful subgroups for further consideration [60]. In recent years, numerical taxonomy techniques have been increasingly used in the study of various birth defects, e.g. agenesis of the penis [74], tracheal agenesis [75] and radial ray deficiencies [76]. Fetal hydrocephalus can occur either as an isolated malformation or together with other birth defects. Several conditions, both syndromic and non-syndromic, include hydrocephalus in their spectrum of anomalies. In this study, numerical taxonomy was used to test the hypothesis that, among those cases of fetal hydrocephalus with associated malformations, distinct subgroups exist.

Seventy-eight (59.5%) of the cases of fetal hydrocephalus in this study had associated malformations. This is in close accordance with the results of a study by Roume et al. who found that in a series of 94 cases of fetal hydrocephalus, 60.6% had associated malformations (excluding NTD) [12]. As detailed in the results section, application of numerical taxonomy to the 78 cases of complex fetal hydrocephalus yielded three distinct subgroups, confirming the third hypothesis of the study. Although pregnancy outcome was not considered in the analysis due to incomplete data, it is worth mentioning that in general there was a worse outcome associated with cases of complex fetal hydrocephalus than cases in which hydrocephalus was an isolated finding. This observation supports the findings of previous studies [8,17].

Of the three clusters identified by the numerical taxonomy, two have strong associations with malformations of particular body systems. Cluster two represents a subgroup of fetal hydrocephalus with other CNS malformations. The pathogenetic

mechanism resulting in these findings may in some cases be an early embryonic insult involving ectodermal differentiation, most likely during neurulation. There are strong associations with malformations of the corpus callosum and cerebellum in cluster two. Some of these cases likely represent instances of Dandy-Walker malformations or variants. As well, three cases in this cluster have Walker-Warburg syndrome. Both Dandy-Walker syndrome [23] and Walker-Warburg syndrome [24] include cerebellar malformations in their spectrum of anomalies.

It is interesting to note, however, that not all CNS malformations clustered into the same subgroup. Malformations of the cerebrum, including holoprosencephaly and lissencephaly, were found predominantly in cluster one. In fact, there is evidence of other "sub-clusters" in cluster one as well, e.g. heart defects. These "sub-clusters" may actually represent distinct clusters that were combined due to the small numbers of individuals involved.

Cluster three represents a subgroup of fetal hydrocephalus with craniofacial and musculoskeletal malformations. Early defects in ectodermal-mesodermal patterning or interaction may be the pathogenetic mechanism responsible for these findings. There is a high incidence of cervicothoracic and lumbosacral spine/rib malformations, micrognathia, and limb and renal defects in this subgroup. Vertebral, limb, and renal anomalies are characteristic of the VACTERL-H syndrome and micrognathia has also been reported in some individuals with this disease [66]. It is not surprising then that five of nine cases of VACTERL-H are found in this subgroup. However, the fact that at least one case of VACTERL-H is found in each cluster underlines the phenotypic heterogeneity of the disease as previously noted by Evans et al. [31]. It is also noteworthy

that this subgroup was associated with the poorest pregnancy outcome. Of the sixteen individuals with a known outcome in this subgroup, only five survived the neonatal period. This may be partly due to the fact that most cases in this cluster had multiple malformations.

The regression equation created in the discriminant analysis correctly classified 94.9% of the cases when predicting cluster membership for the same 78 individuals that were used to create the regression equation. This equation could now be used to classify future instances of fetal hydrocephalus, though it is unlikely that such a high percentage would be obtained using new cases whose cluster membership is unknown [65]. One weakness of the equation is that one of the attributes used as a variable is "Musculoskeletal Unspecified Defect". This vague attribute is not likely to be a useful parameter to help predict cluster membership in future cases.

One potential weakness of this study (and retrospective studies in general) is that the ascertainment of malformations may not have been complete. For example, in cases in which the infant died in the prenatal or neonatal period and an autopsy was not performed, it is impossible to know for certain if all the anomalies present were detected. As well, minor malformations are often not commented upon in hospital charts. These types of errors can have particularly serious consequences in numerical taxonomy analysis, as even minor malformations can influence cluster formation [59]. In the present study it was hoped that careful chart inspection and review of autopsy reports (when available) would minimize such errors.

4.4 Summary

This study consisted of two main parts. The first part dealt with the analysis of MSAFP levels in cases of fetal hydrocephalus. This was the first known population-based study to analyze this relationship. MSAFP levels in cases of fetal hydrocephalus were significantly higher than those of the general population of screened pregnancies ($p = 0.029$). As well, MSAFP levels in the subgroup of cases of complex fetal hydrocephalus were also significantly higher than those of the general population of screened pregnancies ($p = 0.041$).

This study found MSAFP testing to be a poor predictor for fetal hydrocephalus. Only 7 of 70 cases (10%) fell at or above the "elevated" cut-off. However, 6 of the 7 elevated cases (86%) had associated malformations. This suggests that elevated MSAFP levels in pregnancies with fetal hydrocephalus do identify women whose infants are at significant risk to have additional malformations.

The second part of the study dealt with identifying patterns of associated malformations in cases of fetal hydrocephalus. Numerical taxonomy identified three distinct subgroups among the cases of complex fetal hydrocephalus. Cluster one ($n = 42$) was the largest and most heterogeneous of the subgroups. Cluster two ($n = 19$) represented a subgroup of fetal hydrocephalus with other CNS anomalies. Cluster three ($n = 17$) represented a subgroup with multiple malformations. There was a strong association with musculoskeletal and craniofacial malformations and poor pregnancy outcome in this subgroup.

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Appendix 1

MSAFP Levels and Specific Findings in Cases of Complex Fetal Hydrocephalus By Cluster*

Cluster 1

I.D. #	MSAFP	Associated anomalies
1		Polycystic kidney disease, positional anomalies of the limbs, pulmonary hypoplasia, low set ears, erythroid metaplasia of liver. (**Meckel-Gruber**)
59		Macrocrania, mild connective tissue dysplasia, hyperextensibility of joints of hand, long head, prominent ears
58		Vermian hypoplasia, olfactory agenesis, microtia, low set ears
14	0.85	Hypoplastic left heart, single atrial ventricular valve, transposition of the great arteries, bilateral unilobular lungs (**VACTERL-H**)
28	0.45	Hypoplastic left heart, abnormal wrist posturing (**Trisomy 18**)
70	4.3	Patent ductus arteriosus, patent foramen ovale, dysmorphic skull, low set left ear
21		Mild hydronephrosis
52	0.55	Single kidney with hydronephrosis
36		Renal agenesis
33		Hyaline membrane disease, PDA, hyperbilirubinemia, inguinal hernia right side, retrolental fibroplasia
72	1.8	Heart defect
50	0.7	Short limbs (thanatophoric dwarfism)
17	2.3	Cystic hygroma, unilateral pleural left effusion, cardiac anomalies, abnormal limb position, oligohydramnios (**Trisomy 21**)
38		Cystic hygroma, short femurs, renal cystic dysplasia
65	1.5	Blind esophageal pouch, T-E fistula, bilateral hydroureters, bicuspid aortic valve, fusion of mitral commisures, endocardial fibroelastosis of left ventricle (**VACTERL-H**)
15	2.3	Holoprosencephaly, T-E fistula TWIN
19	1.1	Holoprosencephaly

41		Esophageal atresia
60		Adducted thumbs
27	1.7	Adducted thumbs
29	0.54	Contracture of 4th digit on right hand
25		Low set ears, overlapping fingers and toes
66	0.9	Spastic quadriplegia, microcephaly, calcifications of the basal ganglia and thalamus
20		Microcephaly, periventricular calcifications
45		Microcephaly, spastic quadriplegia
62	0.57	Left simian crease
48	1.7	Microcephaly, Skin lesions
42	3.2	Epicanthic folds, hypertelorism, short nose with a long philtrum, hypoplastic toenails
30	0.8	Multiple brain anom., T-E fistula, cong. heart disease, cleft palate
2	1.4	Dysplastic ears, arachnodactyly, anteriorally placed anus, T-E fistula, congenital heart disease (**VACTERL-H**)
5		Cerebral dysplasia
54	0.8	Polygyria
11		Lissencephaly (**Walker-Warburg**)
34		Cerebral atrophy
46		Cerebral atrophy, cortical blindness
64		Loss of cerebral cortex
24	1.0	Increased lung lobation, polygyria
57	1.4	Polygyria, Meckel's diverticulum
39		Diaphragmatic hernia, brochopulmonary dysplasia, AV canal, hypoxic ischemic encephalopathy, cerebral atrophy (**Fryns**)
61	2.9	Cerebral dysgenesis, ascites, cardiac hypertrophy, amputation of fingers both hands, amputation of feet, ambiguous ext. genitalia (**Amniotic Band syndrome**)
31		VSD with PDA and mild concentric hypertrophy of ventricle, dysmorphic facial features, short neck, excess nuchal skin, syndactyly of 2nd and 3rd toes
40	1.6	Patent foramen ovale with oesophageal atresia, right radial and thumb agenesis, left thumb polydactyly, single umbilical artery, incomplete lung lobation, hypoplastic adrenals, straplike ovaries

Cluster 2

I.D. #	MSAFP	Associated anomalies

3		Macrocephaly, dysgenesis of corpus callosum, hypertelorism, antimongoloid eye slant
76		Agenesis of corpus callosum
51	2.2	Agenesis of corpus callosum
22		Contraction of all fingers, agenesis of corpus callosum, cerebral dysplasia
73	2.1	Hypoplastic left heart, T-E fistula with Oesophageal atresia, agenesis of corpus callosum
68		Frontal bossing, hypertolerism, depressed nasal root, short palpebral fissures, anteverted nares, simple helix of ears, macrognathia, polygyria, partial agenesis of corpus callosum, respiratory distress syndrome, hypotension, PDA
9	0.75	Cataracts, deafness, Duchenne muscular dystrophy (**Walker-Warburg**)
8		Hypoplasia of cerebellum, bilateral microphthalmia
56		Micro-opthalmia, camptodactyly of left hand, cataracts (**Walker-Warburg**)
18	0.8	Bilateral cataracts, necrotizing enterocolitis, PDA (**Walker-Warburg**)
63		Abnormal cerebellum
16	0.5	Patent ductus arteriosis
77	0.95	Hypoplasia of cerebellar hemispheres, agenesis of corpus callos.
37		Cerebellar hypoplasia, absent inferior vermis, hypospadias
12	0.61	No cerebellar vermis, microcephaly, agenesis of corpus callosum
49	2.7	Esophageal atresia, T-E fistula, hemivertabrae, absent septum pellucidum, choroid plexus cyst (**VACTERL-H**)
35		Lissencephaly, cerebellar hypoplasia, hypoplasia of brain stem, thin corpus callosum
6	1.4	Microopthalmia, Microglossia, Micrognathia
53		Hypertolerism, down slanting palpebral fissure, carp mouth, right preauricular tag (**Ritscher-Schinzel**)

Cluster 3

I.D. #	MSAFP	Associated anomalies
4		Large anterior and posterior fontanelle, mild hypertelorism, flat nasal bridge, cupped ears, micrognathia, wide spread nipples, left hypoplastic thumb, hypoplasia of radius, sacral sinus, VSD
10	1.3	Alobar holoprosencephaly, digital hypoplasia of hands and syndactyly, webbing of fingers, simian creases, bilateral amput. of both mid-feet w/ absence of toes, hypoplastic mandible

32	1.6	Cleft lip + palate, esophageal atresia, multiple vertebral anomalies, ectopic anus, horseshoe kidney, adducted thumbs (**VACTERL-H**)
74		Cleft lip and palate, skeletal abnormalities (T5 and T7 butterfly vertebrae, bilateral coronal synostosis in the skull, fusion of 1st and 2nd left ribs, 13 ribs bilaterally, 13 thoracic vertebrae)
7		Sacral agenesis, incomplete holoprosencephaly, imperforate anus, vesic-urethral obstruction, rocker bottom feet, hemivertebrae T4 and T5 (**VACTERL-H**)
44		Cleft lip and palate, VSD, imperforate anus, aphallia (**Fetal alcohol syndrome**)
43		Micrognathia, hypoplastic labia majora, hypoplastic lower limbs with paralysis, club feet, mild right nephrosis, 11 pairs of ribs
55		Low set ears, preauricular skin tags, hypoplastic vertebral bodies, Hypoplastic cerebellum and vermis, micrognathia
26		Low set ears, hypertolerism, micrognathia, polydactyly, VSD, patent ductus arteriosus, incomplete horizontal fissure, duplication of jejunum, extra thoracic vertebra with hypoplastic ribs (**Hydrolethalus**)
67	1.5	Low set ears, short webbed neck, cleft palate, hyperextensible knee and elbow, rocker bottom feet, carp mouth, shield chest
69	2.2	Polygyria, disruption of septum pellucidum, absent right hand, hypoplatic right forearm bones, irregular right hemithorax, bilateral Pulmonary hypoplasia, enlarged thymus
23		Hypoplastic lungs, multicystic dysplastic right kidney, dysplatic left kidney, low set ears, hypoplastic left thumb, hypoplastic Metatarsal, sacral dimple, T8 butterfly vertebrae, 13 ribs bilaterally TWIN (**VACTERL-H**)
13	0.94	Renal agenesis, right-sided aortic arch, bilateral cervical, axillae pterygia, facial dysmorphism, preaxial polydactyly of right foot, cleft palate
78	2.6	Agenesis of corpus callosum, hypoplastic cerebellum, abnormal cerebrum, sacral dimple, downslanting palpebral fissures, micropthalmia, micrognathia, thin distally placed thumbs, T-E fistula w/ esophageal atresia, C7 hemivertebra, thoracic scoliosis, abnormal lung lobation, large left liver lobe, renal dysplasia of both kidneys, bilateral hydronephrosis, bilateral hydroureters (**VACTERL-H**)

47		Microcephaly, low set ears, high arched palate, micrognathia, single digital crease right and left fingers, multiple bone anomalies, hypolplastic lungs, dysplastic cartilagenous foci - both kidneys
75	0.85	Small palpebral fissures, upturned nose, small mouth, low set ears, Hypoplastic mandible, webbed neck, hand and feet anomalies, urethral stenosis, bilateral hydroureters, fenestrated AV valve, undescended testes, hypoxic-ischaemic encephalopathy
71	1.3	Low set ears, anteverted nares, wide spread palpebral fissures, cleft palate, small anteriorly tethered tounge, webbed neck, thoracic scoliosis, mild fexion contracture of the elbows, hyperextended wrists, abnormal finger flexion, external rotation of hip, midflexion contracture of knees, valgus rotated left foot, varus rotated right foot, rocker bottom feet, tubular hypoplasia of aortic arch, single left lobed lung, hypoplastic lungs, absent kidneys, Hypoplastic ureters/bladder,no uterus and fallopian tube (**VACTERL-H**)

*Cases shown are in the same order as in the dendrogram.