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A RETROSPECTIVE ANALYSIS OF COMORBID TRAITS AFFECTING FEEDING IN INFANTS WITH DOWN SYNDROME

A Thesis

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of

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Lastly, my fellow namesake should be thanked for her understanding of my need and love of coffee and rice krispie treats. And laughter

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ABSTRACT

Duvall, Nichole L, M.S. Purdue University, December, 2011. A retrospective analysis of comorbid traits affecting feeding in infants with Down syndrome. Major Professor: Randall Roper.

Down syndrome (DS) is the most common aneuploidy to affect humans and occurs in approximately 1 of 750 live births. Individuals with DS present with a wide range of clinical phenotypes. Common craniofacial phenotypic expressions include a small mandible, protruding tongue, and a flattened nasal bridge. These traits may affect the feeding, breathing, and swallowing of individuals with DS. Because some complications may go unnoticed for longer periods of time, we hypothesize that significant cardiac and GI defects may be indicative of feeding and airway difficulties. In order to better understand the secondary phenotypes resulting from DS, we have implemented a retrospective chart review of 137 infants between zero and six months of age who were evaluated through the Down Syndrome Program at Riley Hospital for Children from August 2005 to August 2008. Data regarding cardiac, gastrointestinal, endocrine, airway, auditory, and feeding abnormalities have been collected and incedences and comorbidites of these traits has been examined.

Comprehensive results indicate cardiac abnormalities occur in 80% of infants, 60% experience gastrointestinal complications, feeding difficulties occur in 46%, and

airway complications occur in 38% of infants. Infants with DS were found to be breastfed less over time, with an increase in tube feeds. Notably, we have found all infants with videofluoroscopic evaluations had some type of dysphagia. The presence of gastrointestinal abnormalities closely correlate with the need for tube feeds, and the comorbidity between GI anomalies and muscle tone appear to indicate the likelihood of feeding difficulties and need for altered feeding strategies. Comorbidities between feeding difficulties were nearly significant with cardiac defects and significant with GI abnormalities. Identification of such associations will help healthcare providers determine the best course of treatment and recommended feeding methodology for infants with DS.

In order to utilize an *in vitro* model to study the craniofacial dysmorphologies seen in individuals with DS, cranial neural crest cells (NC) have been cultured. With these, we have begun to investigate the mechanisms behind a smaller trisomic mandibular precursor as compared to the euploid. With this *in vitro* model, we will be able to test proliferation, migration, and senescence of NC in a culture system.

CHAPTER 1. INTRODUCTION

1.1. Etiology of Down syndrome

Down syndrome (DS or Trisomy 21) is the result of three copies of human chromosome 21 (Hsa21) (LEJEUNE *et al.* 1959) and occurs in approximately 1 of 750 live births, making DS the most common human aneuploidy (CHRISTIANSON 2006; SCHIEVE *et al.* 2009; WEIJERMAN *et al.* 2008). Cytogenetic studies have shown that approximately 95% of all DS cases are the result of meiotic nondisjunctions, the majority of which occur during meiosis I in the oocyte (ALLEN *et al.* 2009; HASSOLD and HUNT 2001). The remaining 5% of cases result from Robertsonian translocations (4%) and mosaicism (1%) (BANDYOPADHYAY *et al.* 2003; FREEMAN *et al.* 2007; HASSOLD and HUNT 2001). Specifically, Robertsonian translocations refer to the whole-arm rearrangement of chromosomes, usually where the long arms of Hsa14 and 21 break at the centromere and attach to one another (BEREND *et al.* 2003; MUTTON *et al.* 1996). Mosiacism occurs as only a portion of the cell population receives the third copy of Hsa21 while the remainder is euploid (BANDYOPADHYAY *et al.* 2003). It has also been noted that the incidence of DS births increases with advanced maternal age (ALLEN *et al.* 2009; CHRISTIANSON *et al.* 2004; SHERMAN *et al.* 2005).

1.2. Phenotypes of Down syndrome

1.2.1. Craniofacial dysmorphology

Craniofacial abnormalities occur in essentially all individuals with DS, though the severity of the phenotypes vary between individuals (ANTONARAKIS *et al.* 2004; CLEVES *et al.* 2007; SHERMAN *et al.* 2007). Common craniofacial phenotypic expressions include brachycephaly, shortened midface, small mandible, protruding tongue, and a flattened nasal bridge (EPSTEIN 2001a; SHOTT 2006a; VAN CLEVE and COHEN 2006). Together, these structural anomalies can create functional feeding and airway difficulties in individuals with DS (COOPER-BROWN *et al.* 2008; FAULKS *et al.* 2008; HILLEMEIER *et al.* 1982; MARCUS *et al.* 1991; ROIZEN 2003a; SHOTT 2006a). Airway difficulties most often include obstructive sleep apnea (OSA) and upper airway occlusion (UAO), which are collectively reported to occur in approximately 50-75% of all individuals with DS (MARCUS *et al.* 1991; ROIZEN 2003a; SHOTT 2006b).

Obstructive sleep apnea syndrome (OSAS) includes multiple deficiencies such as complete or partial apneas or hypopneas, hypoventilation, and blood-oxygen desaturation. These can all result in neurodevelopmental problems including daytime somnolence, behavioral problems, school failure, and developmental delay. The numerous phenotypes seen in DS, such as the underdevelopment of the midface and mandible, small upper airway, hypotonia, and superficially positioned tonsils, all lead to an increased predisposition to OSAS and UAO (BROUILETTE *et al.* 1984; ROIZEN 2003a).

Polysonograms (PSGs) are most often the method of OSA and UAO diagnosis. Marcus *et al.* found PSGs were abnormal for 77% of individuals with DS, and 45% were diagnosed with OSA. Hypoventilation was observed in 66% of the individuals and oxygen desaturation was present in 32%. Of the 77% with abnormal PSGs, 63% also had either hypoventilation or oxygen desaturation. Interestingly, in a separate study, 16 children underwent both nap and overnight PSGs and although the nap study revealed 75% had an abnormal PSG, all the children had abnormal overnight PSGs. This demonstrated that nap PSGs can sometimes underestimate the severity, or even presence of, sleeping anomalies and it should therefore be recommended that overnight PSGs be used as a method to investigate abnormal airway function during sleep (MARCUS *et al.* 1991).

Additionally, another study by Levanon *et al.* also revealed the underestimation of severity a PSG may provide. Here, 23 children with DS underwent overnight PSG and only 4 were diagnosed with OSAS, which was not significantly different than the control group. However, they did demonstrate that children with DS have sleep abnormalities characterized by increased fragmentation of REM sleep, numerous awakenings and arousals, and periodic leg movements. While not OSAS, these disruptions to a good nights' rest can impede the development of a child and their ability to thrive (LEVANON *et al.* 1996; SHOTT 2006b). Together, these studies demonstrate that not only are individuals with DS have difficulties breathing while sleeping as a result of craniofacial dysmorphologies, but that these complications can cause problems in behavior and development.

1.2.2. Cardiac defects

Individuals with DS present with a wide range of clinical phenotypes but most often noted are structural abnormalities such as congenital heart defects (CHDs) and gastrointestinal defects, including Hirschsprung disease (ANTONARAKIS *et al.* 2004; CLEVES *et al.* 2007; EPSTEIN 2001a; PATTERSON 2009; ROPER and REEVES 2006; WEIJERMAN *et al.* 2008). In recent years, the echocardiogram (EKG) has been the gold standard for cardiac anomalies early in life if the newborn is suspected to have DS, thereby making the diagnosis of a CHD more precise (WELLS *et al.* 1994). The Atlanta Down Syndrome Project (ADSP) and its successor, the National Down Syndrome Project (NDSP) are the largest population databases to combine infant medical records, maternal questionnaires, and DNA methodology in order to study the etiology of DS. From the NDSP, cardiac defects are reported to occur in 44% of the DS population (FREEMAN *et al.* 2008; FREEMAN *et al.* 1998).

Within the population of those with DS and congenital heart defects, isolated endocardial cushion anomalies (sometimes atrioventricular septal defects AVSDs, atrioventricular canal defects, and hereafter AVCDs). AVCDs alone or in combination with another abnormality are found to be present in 31-43% of individuals with DS (FREEMAN *et al.* 2008; PARK *et al.* 1977; TORFS and CHRISTIANSON 1998). Ventricular septal defect (VSD) are found in approximately 32-43% (FREEMAN *et al.* 2008; PARK *et al.* 1977) of individuals with DS. Atrial septal defects (ASDs) are reported as being 39-43% (FREEMAN *et al.* 2008; STOLL *et al.* 1998) of the DS population with CHDs. Berg *et al.* were one of the first to designate the presence of a patent foramen ovale (PFO) was not pathological, nor was a patent ductus arteriosis (PDA) before 3 months of life, but

rather an artifact of fetal circulation (BERG *et al.* 1960; PARK *et al.* 1977). Patent ductus arteriosis (PDA), when included, account for approximately 4% while the tetralogy of Fallot (TOF) is estimated to occur in 6% of those with DS (BERG *et al.* 1960; FREEMAN *et al.* 2008; PARK *et al.* 1977; TANDON and EDWARDS 1973; WELLS *et al.* 1994).

1.2.3. Gastrointestinal defects

Most reports of gastrointestinal (GI) abnormalities involve the incidence of Hirschsprung disease in the DS population. In 2009 the NDSP provided one of the most comprehensive accounts of occurrences of GI defects in individuals with DS. The most reported GI abnormalities include esophageal atresia, tracheoesophageal fistula, pyloric stenosis, duodenal stenosis/atresia, annular pancreas, Hirschspung disease, and anal stenosis/atresia together at a maximum occurrence of 6.8% (FREEMAN *et al.* 2009). They also found no significant association between GI defects and infant sex, ethnicity, or maternal age. Duodenal atresia is the most common GI defect seen in DS with rates between 1-5%. They found a 0.85% occurrence rate of Hirschsprung, which is similar to the previously recorded rate. It may also occur more often in males, infants of young mothers, and in black infants, but rates are not significantly different (FREEMAN *et al.* 1998; FREEMAN *et al.* 2009).

1.3. Feeding in individuals with Down syndrome

It appears that structural defects in individuals with DS is represented considerably more often in the literature, most often cardiac and GI abnormalities.

However, the functional anomalies that individuals with DS experience can create difficulties as well. Therefore, it can be beneficial to understand the prevalence of feeding complications. And yet, relatively few studies have exclusively examined the feeding difficulties faced by individuals with DS. Dysphagia is defined by difficulties in eating, drinking and swallowing. As high as 80% of individuals with DS are affected by dysphagia as a result of a combination of structural and medical complications (FIELD *et al.* 2003; LAZENBY 2008). Structural complications can include underdevelopment of the facial mid-third as seen in individuals with DS, in addition to a small oral cavity and a narrow, short palate (COOPER-BROWN *et al.* 2008; FAULKS *et al.* 2008). Individuals with DS often exhibit multiple complications that can interfere with feeding such as hypotonia, oral-motor difficulties, reduced suckle ability, weak lip seal, reduced suck/swallow reflex, poor coordination, and difficulties maintaining a consistent sucking rhythm (FAULKS *et al.* 2008; FRAZIER and FRIEDMAN 1996; ZARATE *et al.* 2001).

As infants, these oral complications can cause it to be extremely challenging to breastfeed and the protective role of breast milk against disease can provide assistance to a population with a high risk for many health problems including infections, immune system disorders, and obesity (FLORES-LUJANO *et al.* 2009; PISACANE *et al.* 2003). When questioned in regards to their child's ability to successfully breastfeed, nearly half of a cohort of 59 women reported the infant displayed difficulties in the initiation and continuation of suckling. It was suggested that with enough perseverance and hospital support that the infant with DS can successfully breastfeed (AUMONIER and CUNNINGHAM 1983). Later, in a study by Pisacane in 2003, it was found that the prevalence and duration of breastfeeding among infants with DS were significantly lower

with a mean duration of 54 days, as compared to 164 days in infants without DS. Additionally, reasons for not breastfeeding given by mothers including personal frustration and the infants' difficulty in suckling (PISACANE *et al.* 2003). Together, these investigations bring to light the difficulties infants with DS have with breastfeeding, but only from a mother's point of view. These cannot take into account the lack of discomfort individuals with DS display (FAULKS *et al.* 2008; LAZENBY 2008) and thereby difficulties that can only be diagnosed by evaluations by medical professionals such as penetration and aspiration of feeds.

Individuals with DS not only have difficulties in breastfeeding, but rather complications with feeding in general can cause discomfort and result in aspiration, poor nutrition, and a failure to thrive (COOPER-BROWN *et al.* 2008; FIELD *et al.* 2003; SPENDER *et al.* 1996). The use of videofluoroscopic evaluations, especially in infants, can reveal feeding complications that may not be apparent in individuals. In a retrospective study by Frazier and Friedman in 1996, videofluoroscopic modified barium swallows (VMBS) were utilized to investigate the swallow function in children with DS aged 3 months to 3 years. During the VMBS, swallow function was evaluated by oral preparation (including speed and thoroughness of chewing and bolus cohesion), reflex initiation, pharyngeal clearing, aspiration, and cricopharyngeal screening. For those using a bottle, observations included nipple placement, efficiency of expulsion of liquid from the nipple, and maintenance of a consistent sucking rhythm. In the young infants, oral-motor problems included difficulty with suck initiation, weak lip seal, decreased suction on the nipple, fatigue, and difficulty coordinating suck/swallow/breathing. In addition to oral hypotonia, individuals were found to experience oral sensitivity problems, including oral

hypersensitivity. Most infants demonstrated a slow initiation of a swallow response and displayed aspiration of feeds, of which a few had GER or a cardiac defect, and all children who aspirated and had information regarding coughing were noted to be silent aspirators (FRAZIER and FRIEDMAN 1996). These complications can create discomfort for the individual and inhibit their ability to thrive, and again, may not be diagnosed without the use of video evaluations.

Children with DS not only have difficulties in suckling and lip seal while feeding as an infant, but feeding continues to be difficult as the child ages for reasons including continued weak musculature and difficulty to chew and form a proper bolus (COOPER-BROWN *et al.* 2008). In a study by Spender *et al*. in 1996, it was found that individuals with DS have significantly greater oral-motor dysfunction and an increased tendency to have difficulty with solid foods than a comparison group of children without DS. The individuals also displayed a delayed acceptance of food, poorly coordinated movement of food from the lips into the pharynx when eating pureed or solid textures, delayed initiation of feeding sequences for solid and cracker textures, and an overall decreased control of the jaw. Together, these represent aspects of impaired muscle coordination unique to children with DS (SPENDER *et al.* 1996). Later in life, many individuals still have problems with control of jaw movements, abnormal chewing cycles, and potential difficulties in swallowing coordination. Persistent problems can then become habitual if not corrected early in the child's life, and can lead to behavioral issues that can not only be difficult for the individual himself, but can also produce problems in social integration (FAULKS *et al.* 2008; LAZENBY 2008).

In additional to functional complications that individuals with DS face when eating, behavioral and motivational factors often affect the individual feeding (FIELD *et al.* 2003; SPENDER *et al.* 1996). Skill-based problems such as difficulties in sucking, chewing, or swallowing are a result of genetic and developmental issues, which can prevent them from learning the appropriate way to consume food. Motivational problems stem from an environment that often coaxes or begs a child to eat, or in the least, draws attention to a refusal to eat, and can encourage inappropriate eating habits. In a study by Field *et al.* in 2003, it was demonstrated that children with DS had a significantly greater selectivity for texture when compared to children without DS (FIELD *et al.* 2003). Receiving the proper nutrition and the ability to thrive is a constant concern for parents of children with DS. Parents were found to be more likely to supervise feeding in a directive but non-verbal way, and less likely to facilitate feeding. When asked about any feeding difficulties in their children, nearly half did not notice any complications, and overall, parents were more likely to overlook difficulties or attribute them to food fads or naughtiness (SPENDER *et al.* 1996).

1.4. Healthcare guidelines for individuals with Down syndrome

Clinical features found in individuals with DS are well documented in the literature and include, but are not limited to, cardiac defects, GI abnormalities, thyroid disease, otitis media, obstructive sleep apnea, eye disease, hearing loss, and celiac disease. Recognition of these difficulties that individuals with DS face allows for the specific care of these complications, even as infants and children. It is this understanding

of complications and the need for early treatment that drives the existence of specific health care guidelines for this population. In 2008, Davidson expressed the importance of comprehensive guidelines for individuals with DS in order to anticipate health care issues and screen early and effectively (COHEN 1999; DAVIDSON 2008; ROIZEN 2003a; VAN CLEVE and COHEN 2006).

The current health care guidelines for individuals with DS were published by the Committee on Genetics of the American Academy of Pediatrics (AAP) in 2001 and are significant because they serve as the national standards for caring for individuals with DS. During the neonatal period, from birth to one month, the guidelines focus on the parents' understanding of the DS diagnosis and possible occurrences to watch for when at home (COHEN 1999). First and foremost, it is recommended that clinicians should discuss their patients with DS with a balanced point of view. During the first routine visit clinicians should discuss problems that may present during infancy and what to look for in the child (VAN CLEVE and COHEN 2006).

After an initial meeting with the parents to ensure they understand what a diagnosis of DS can mean and a general explanation of what they can expect, the first clinical examination can be helpful in aiding the medical professional in the care for a specific child. In the examination of the child, it is recommended that the medical professional pay special attention to cardiac difficulties, including an echocardiogram evaluated by a pediatric cardiologist, even in the absence of a murmur. The guidelines, however, do not specify an additional echocardiogram if an EKG was performed on the first day of life. It is also stressed that the medical professional pay attention to any otolaryngological issues the child may be having, including the lack of a red reflex, otitis

media, and infants should be referred for auditory brainstem response (ABR) or otoacoustic emission (OAE) tests within the first three months (COHEN 1999). Furthermore, in any physical examination, areas such as gastrointestinal, heart, and muscle tone should be monitored closer than for children without DS (VAN CLEVE and COHEN 2006).

For children with DS of one to twelve months of age, it is recommended that medical professionals be wary of respiratory problems and dietary management. In the examination, general neurological, neuromotor, and musculoskeletal assessments are made in addition to otolaryngological issues and possible referral to an ENT. Echocardiograms are recommended if not done in the first month, as well as ABR and OAE tests. Early intervention should be discussed for physical and occupational development (VAN CLEVE and COHEN 2006).

Despite the information describing the difficulties children with DS experience when feeding, very little is mentioned concerning health care guidelines in the first year of life. The official guidelines recommend taking notice of constipation and the possibility of Hirschprung disease in the first six months of life (COHEN 1999). Davidson notes in her study of primary care that mothers may need additional support by health care professionals for nursing to be successful. The lower resting metabolism and shorter stature of individuals with DS can result in obesity when compared to "typical" children and therapy to stress healthy eating habits and increased mobility is recommended early in life (DAVIDSON 2008). Therefore, height and weight in children with DS should be compared to both DS height and weight charts and normal growth charts. It is not enough to say that a child can fall in a "normal" range for those with DS, but rather,

whether they are at a healthy height and weight for their age. Continuous monitoring of eating, gastrointestinal, and any sleeping problems should be continued through late childhood until norms for the child can be determined (VAN CLEVE and COHEN 2006).

1.5. Current comorbid and correlation studies

The interest in mapping correlations within DS most often lies in the relationship between genotype and phenotype (DELABAR *et al.* 1993; KORENBERG 1990; KORENBERG *et al.* 1990a; WISEMAN *et al.* 2009). Often, relationships involving DS relate the diagnosis itself to another disorder. Correlations are repeatedly found between a DS diagnosis and the increased risk of developing Alzheimer disease and present with phenotypes such as dementia and amyloid plaques found in the brain (BEACHER *et al.* 2009; MANN 1988; NOBLE 1998). Celiac disease screening is often recommended following a DS diagnosis (COHEN 2006; PAVLOVIC *et al.* 2010). Various forms of thyroid dysfunction often overlap with several difficulties experienced by individuals with DS such as hypotonia, cognitive impairment, and obesity, causing further complications (KAPUR *et al.* 1998; ROSEN 2010; SHAW *et al.* 2006; SKJODT *et al.* 1999). It was also found that in individuals with DS with some type of thyroid dysfunction had a lower development quotient, as a measure of muscle tone, as compared to those without thyroid problems (SHAW *et al.* 2006). Additionally, many researchers have found a relationship between DS and both acute myeloid and lymphoblastic leukemia (FLORES-LUJANO *et al.* 2009; TIGAY 2009; ZWAAN *et al.* 2008). These examples, while helpful, are well documented unlike the specific comorbidities of phenotypes found within DS.

Knowledge of the comorbidity of clinical features seen in individuals with DS can provide improved health care for these individuals from an understanding of what could be expected. Few studies have focused on the correlations between clinical features, but rather exist, if at all, as a side note within an exploration of a specific phenotype. In a study by Freeman *et al*. in 2008, correlations were examined by the National Down Syndrome Project when comparing cardiac defects with gender, ethnicity, and maternal age. It appears that in individuals with DS, ventral septal defects (VSD) occur more often in children born to mothers of ages greater than 35 than to younger mothers. Additionally, white females were almost twice as likely to have an atrioventricular septal defect (AVSD) than any other gender or ethnicity (FREEMAN *et al.* 2008). However, Park *et al.* found individuals with DS with a cardiac defect to have a 20% chance of having another anomaly, most often a structural defect in the gastrointestinal tract (PARK *et al.* 1977).

Additional analyses of co-occurrences between GI abnormalities and other DS-related anomalies most often result in a high incidence of cardiac-GI comorbidity ranging between 50-57% (FREEMAN *et al.* 2009; HILLEMEIER *et al.* 1982; ROWE and UCHIDA 1961; TORRES *et al.* 1998). However, these analyses are limited by the inclusion of only well documented structural defects such as cleft palates or cataracts. Functional difficulties can create problems for individuals with DS as well, though they are not as well recognized. Hillemeier *et al.* presented several cases studies in 1982 documenting patients with DS that had some form of esophageal dysfunction, including those with gastroesophageal reflux (GER). It was discussed that seemingly innocuous symptoms such as acidic reflux may be treated successfully with antireflux therapies, but GER could

be a sign of a larger esophageal dysfunction as it can be related to the inability of the esophagus to clear acid, possibly due to reduced peristalsis (a possible shortcoming from hypotonia in individuals with DS). Constipation, as another functional anomaly that can result from GI abnomalities, is rarely mentioned in the literature except to clarify that it is not abnormal in infants and is expected in newborns with DS (HILLEMEIER *et al.* 1982). Together, both GER and constipation could be indicative of a larger problem.

Comordities regarding feeding in individuals with DS can be extremely beneficial in the health care of this population because early screens for feeding difficulties are not yet a part of the national health care guidelines. Cardiac anomalies are sometimes associated with complications in breastfeeding for infants with DS, including poor suckling abilities (AUMONIER and CUNNINGHAM 1983; SAENZ 1999; SHAPIRO *et al.* 2000). The presence of gastrointestinal abnormalities, especially GER, are also related to feeding difficulties (BUCHIN *et al.* 1986; FIELD *et al.* 2003; ZARATE *et al.* 2001). While some problems may not be specific to DS, the high incidence of GI abnormalities seen in DS could provide reason to screen early for feeding complications if a GI abnormality is discovered (FIELD *et al.* 2003). Lastly, hypotonia is generally recognized as being a major contributor to the difficulty individuals with DS experience while feeding (MIZUNO and UEDA 2001; SPENDER *et al.* 1995; SPENDER *et al.* 1996; VAN CLEVE and COHEN 2006).

1.6. Mouse models of Down syndrome

A useful tool for studying any genetic disorder includes a model system. Substantial conservation in gene content and order occurs between human and mouse (GARDINER *et al.* 2003; HATTORI *et al.* 2000; MURAL *et al.* 2002). Numerous phenotypes associated with DS are also apparent in mouse models (recently reviewed in (DELABAR *et al.* 2006; MOORE and ROPER 2007; WISEMAN *et al.* 2009)). Mouse models have therefore been effectively used to understand the gene-phenotype relationship in DS.

The earliest murine model for trisomy, the Ts16 mouse, had a complete third copy of Mmu16, which not only created an inaccurate gene dosage imbalance, but mice did not survive past birth making it difficult to use for postnatal development studies (DAVISSON *et al.* 1993; GROPP 1982). Chimeric mice were used for a time, but were also difficult to study due to inconsistent severities of phenotypes and the inability to generate reproducible results (EPSTEIN *et al.* 1982). Therefore, segmental trisomies were created in mice that triplicated portions of the murine genome to artificially reproduce Ts21(DAVISSON *et al.* 1990). The most widely used and well studied mouse model of trisomy and DS phenotypes is the Ts(17^{16})65Dn (hereafter Ts65Dn). Davisson *et al.* created the Ts65Dn mouse using mice that were heterozygous for reciprocal translocations (DAVISSON *et al.* 1993). This segmental trisomy model has a small translocation chromosome comprised of the distal region of Mmu16 just proximal to the *App* gene attached to the centromeric end of Mmu17 (DAVISSON *et al.* 1993; REEVES *et al.* 1995) and contains approximately half of the Hsa21 gene orthologs (HATTORI *et al.* 2000). Ts65Dn mice show DS-related phenotypes including reduced birth weight, perinatal lethality, craniofacial abnormalities, cognitive and behavioral impairments,

cardiovascular malformations, and neurological structural deficiencies (BAXTER *et al.* 2000; BELICHENKO *et al.* 2004; COOPER *et al.* 2001; HOLTZMAN *et al.* 1996; MOORE 2006; RICHTSMEIER *et al.* 2000; ROPER *et al.* 2006; RUEDA *et al.* 2005).

1.7. Development and the role of the neural crest

The neural crest is a transient structure consisting of highly pluripotent cells that yield a number of cell types including bone, tendon, connective and adipose tissue, melanocytes, neurons, and endocrine cells (CHARRIER *et al.* 2005; KNIGHT and SCHILLING 2006; LE DOUARIN *et al.* 2004; LE DOUARIN and DUPIN 2003; SANTAGATI and RIJLI 2003). In order to study the specific dysmorphologies of the craniofacial skeleton as seen in both humans with DS and the corresponding mouse models, the overall development of the cranium should be investigated. The cranial neural crest cells (CNCC) contribute to several structures in the head and neck region including the skeleton of the cranium, dermis, smooth muscles, and connective components of striated muscles in the head, ocular and periocular tissues, the pia-arachnoid membrane, root ganglia of cranial nerves IX and X, and possibly an initial promoter of tooth development (CHAI *et al.* 2000; CHARRIER *et al.* 2005; COULY *et al.* 1998; KURATANI 2009; LE DOUARIN 2008). During development, the CNCC emerge from the dorsal neural tube and migrate in a conserved pattern as one of three streams: trigeminal (mandibular), hyoid (preotic), and branchial (postotic) (KNECHT and BRONNER-FRASER 2002; KNIGHT and SCHILLING 2006). The CNCC that migrate as the trigeminal stream will contribute to

the first branchial arch that will contribute to the mandible, palate, tongue, and the bones of the middle ear (LE DOUARIN 1999; SANTAGATI and RIJLI 2003).

It has been established that individuals with DS have small mandibles and, therefore, small oral cavities, which contribute to the functional difficulties individuals with DS experience by way of feeding and breathing (CLEVES *et al.* 2007; EPSTEIN 2001a; MIZUNO and UEDA 2001; ROIZEN 2003a). In Ts65Dn mice, it has been demonstrated that the trisomic mandibular precursor, the first branchial arch (BA1), is smaller both in volume and number of CNCC than euploid littermates at embryonic day 9.5 (E9.5). Additionally, it was found that there were fewer CNCC that delaminated from trisomic neural tube explants and migrated a shorter distance than CNCC from euploid embryos (ROPER *et al.* 2009). Together, the reduced migration and proliferation of the trisomic CNCC as compared to the euploid might explain the smaller BA1, and postnatally the smaller mandible, which could be utilized to further understand how the differences in craniofacial development lead to functional difficulties experienced by individuals with DS.

In addition to the CNCC, cardiac, vagal, and trunk neural crest migrate away from the neural tube to populate developmental structures more posterior to those contributed to by the CNCC (KURATANI 2009; LE DOUARIN 1999). Cardiac NC originate from somites 1-4 and populate branchial arches 3,4 and 6, where the BA4 contributes most highly to the aorticopulmonary and conotruncal septa of the heart (CHARRIER *et al.* 2005; LE DOUARIN 2008; STOLLER and EPSTEIN 2005). Ectomesenchymal cells from the cardiac crest are involved in the development of smooth muscles in the great arteries, thymus, thyroid, and the endocardial cushions. These cushions will contribute to the

outflow tract of the heart. Cardiac neural crest also provide the parasympathetic innervations of the heart, and contribute to the semilunar and atrioventricular valves, but the function of the crest within the valves in unknown (LE DOUARIN 1999; STOLLER and EPSTEIN 2005). Vagal NC arise from somites 1-7 and colonize the entire length of the gut that later play a role in the development of both the gut and enteric nervous system. The trunk NC, from the caudal end to somite 28, contribute to the sympathetic nervous system innervations of the postumbilical portion of the bowel and melanocytes (CHAI *et al.* 2000; LE DOUARIN 1999; LE DOUARIN 2008; RICHTSMEIER *et al.* 2002).

1.8. Hypotheses

Structural abnormalities such as cardiac or GI defects are often detected early in individuals with DS by medical professionals. The healthcare guidelines recommend EKGs for all infants, and further investigation of infants with GI mobility difficulties. However, many cases of functional difficulties such as feeding or airway complications may be overlooked. Infants may be silently aspirating during feeding and most people feel snoring during sleep is normal. This further indicates the need for early screening of functional complications as they may not be diagnosed until later in life. Therefore, in collecting data regarding clinical traits found in infants with DS, I hypothesized correlations between the presence of cardiac and GI abnormalities with the occurrences of feeding and airway complications. I hypothesize cardiac and GI abnormalities will often co-occur with feeding and airway anomalies. This information could help highlight the need for early functional screens. Additionally, with the compilation of detailed

feeding data, I plan to elucidate the greater feeding complications experienced by infants with DS.

The functional difficulties faced by individuals with DS stem from a structural component. Both a small mandible and oral cavity contribute to feeding and airway complications. At E9.5 the mandibular precursor was found to be smaller and populated with fewer NC in trisomic murine embryos as compared to euploid littermates. By culturing CNCC and examining the proliferative and senescent qualities, I will utilize them as an *in vitro* model to understand the possible mechanisms behind the size differences in trisomic and euploid BA1s. I hypothesize that culturing NC in an *in vitro* system could be used to investigate the possible differences in proliferation, migration and senescence of trisomic and euploid cells.

CHAPTER 2. INTRODUCTION

2.1. Retrospective data collection

Data for this study were collected in a retrospective chart review of 137 infants between zero and six months of age who were evaluated through the Down Syndrome Program and Riley Hospital for Children from August 2005 to August 2008. Information was collected regarding cardiac, GI, feeding, airway, auditory, and endocrine systems as documented in medical records. Only information within the first six months of life was entered into an Excel spreadsheet with no identifying information. Specifics of data collection included: gestational age, birth weight and length, Apgar scores, ethnicity, type of trisomy (standard, Robertsonian translocation, or mosaism), results of physical at visits, absence or presence and type of CHD and any related medication or surgery, oximetry swallow studies and complications found, feeding route and type at birth and subsequent visits, polysonograms and associated problems, auditory testing including newborn hearing screen, ABRs and OAEs, and any thyroid abnormalities.

This study was in accordance with an exempt IRB protocol reviewed and approved by the Indiana University School of Medicine and data collection followed HIPAA guidelines. To ensure the privacy of patients, personal identifiers were not recorded and individuals were represented as numbers. No records of number-patient connection were kept. Data was collected from a chart in a separate room to ensure the

privacy of a patient. Additionally, to reduce the influence of personality on data collection, only three individuals recorded data, and one researcher was present for collection of nearly all medical records to aid in any discrepancies.

Birth information was collected in an Excel spreadsheet where data regarding gender, karytyope and ethnicity was also recorded (Table 1). Physical data was recorded from every clinic visit including weight and height (Table 2). The type of cardiac defect was recorded, as well as if it had been repaired or required any type of medication (Table 3). Both structural and functional GI abnormalities were recorded. Repairs were noted, as were any additional medications (Table 4).

Feeding information from parents was collected including the route and type of feed. Data were also taken from feeding evaluations, most often a clinical swallow study, where any complications were recorded (Table 5). Data from polysonograms were recorded from test result (Table 6). Newborn hearing screen results were collected, as well as any additional exams, such as the OAE and ABR (Table 7).

Lastly, the gross motor developmental quotient was calculated using the gross motor skill level, found in the clinic visits, and corrected age (Table 8). The gross motor skill level must be available for two visits to find the DQ, and was often difficult to ascertain. The DQ was calculated as follows:

$$\frac{\text{Gross motor skill level at Time 2 (in months)}}{\text{Corrected age at Time 2 (in months)}}$$

2.2. Statistical analysis

Differences regarding feeding strategy and either the presence or absence of a cardiac or GI abnormality were examined using either the Chi-square (χ^2) Goodness of fit, Fisher's exact test, or an Odds ratio. A χ^2 test was used if there were zero individuals in a particular comparison group, where a Fisher's exact test was utilized if there were individuals to represent all groups in a 2x2 comparison table. The Odds ratio was utilized only if the 2x2 table could be filled and provided a fold difference between comparison groups.

2.3. Ts65Dn breeding

Female B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn) and male B6 x C3H/HeJ F_1 (B6C3F1) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Wnt1-Cre mice came from the lab of Dr. Yang Chai of the University of Southern California and backcrossed >6 generations to C57BL/6J (B6) mice. Offspring from Ts65Dn x B6C3HF1 matings were produced in our colonies at Indiana University-Purdue University Indianapolis (IUPUI). All male/female pairs are kept in a 12 hr day/12 hr night light cycle with food and water administered *ad libitum* in a temperature and humidity controlled room. Animal use and protocols were reviewed and approved by the IACUC committee at IUPUI.

2.4. Genotyping of Ts65Dn mice

2.4.1. Polymerase Chain Reaction

PCR was utilized to amplify a 246 bp segment with a single nucleotide polymorphism (SNP) in the *Zdhhc14* gene (rs48029645 GenBank) using the primers (forward) 5'-AAATAGTAGCATCTCATGAGTG-3' and (reverse) 5'-CATAGTGCATCTTAGACAAGC-3'(T_m=60°C) (Invitrogen, Carlsbad, CA) (Figure 1). For a single reaction, 1X PCR Rxn Buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), and 0.02 μg/μL TaqDNA Polymerase (Bioline, Taunton, MA) were used with 2 μL of DNA (approximately 100 ng/μL) in a 25 μL reaction. DNA was amplified using the following steps: 1) 94°C for 1 min, 2) 94°C for 30 sec, 3) 65°C for 20 sec, 4) 72°C for 20 sec, 5) repeat steps 2-4 5 cycles, 6) 94°C for 30 sec, 7) 65°C for 30 sec (-1 degree per cycle), 8) 72°C for 1 min, 9) repeat steps 6-8 10 cycles, 10) 94°C for 30 sec, 11) 55°C for 30 sec, 12) 72°C for 20 sec 13) repeat steps 10-12 10 cycles,14) 72°C for 1 min, 15) 72°C hold. Afterwards, 8 μL of the PCR can be removed and a 246 bp band visualized on a 1% agarose gel.

2.4.2. Restriction digest

The *Sac*I enzyme was used to discern the SNP in PCR products. For a single reaction, 2 μL of Buffer I (New England Biolabs, Ipswich, MA), 2 μL of 10X BSA, 7 μL sterile water, and 1 μL of *Sac*I restriction enzyme were combined in a 1.5 mL eppendorf tube to which 16 μL of the PCR product was added. Samples were incubated in a 37°C

water bath for 2.5 hours and resolved on a 3% agarose gel. Products were visualized using SYBR Safe DNA gel stain (Invitrogen) and a UV source (LORENZI *et al.* 2010; LORENZI 2006).

2.5. Fluorescence in situ hybridization (FISH)

Fluorescence *in situ* hybridization was utilized to genotype adult mice using blood samples taken from the tail, or embryos using yolk sacs obtained during a dissection (MOORE *et al.* 1999; ROPER *et al.* 2009).

2.5.1. Preparation of embryonic yolk sacs

Yolk sacs were removed into 0.5 mL Dulbecco's PBS (DPBS) (Mediatech, Herndon, VA) and centrifuged at 12,000 rpm for 1 minute. The supernatant was removed, pellet resuspended in an additional 0.5 mL DPBS, and centrifuged again for 1 minute at 12,000 rpm. The supernatant was removed, pellet was resuspended in 0.5 mL Collagenase XI-S (1000 u/mL in HBSS) (Sigma, St.Louis, MO), and incubated at 37°C for 30 minutes. Following the incubation, the yolk sacs were centrifuged again at 12,000 rpm for 1 minute, the supernatant was removed, and the pellet resuspended in 0.5 mL 0.075M KCl (Gibco, Grand Island, NY). Yolk sacs were vortexed briefly and allowed to incubate for 30 minutes at 37°C. After the incubation, 1 drop of 3:1 methanol:acetic acid was added to each yolk sac, vortexed, then centrifuged at 12,000 rpm for 1 minute. The supernatant was removed, pellet resuspended in 3:1 fix, and refrigerated for 24 hours.

2.5.2. Preparation of FISH slides

Yolk sacs that have been refrigerated overnight in 3:1 fix were centrifuged at 12,000 rpm for 5 minutes. All but 100 μL for supernatant was removed and pellet was resuspended. Using a glass pipette, a single drop of water was added to two labeled VCE slides, followed by alternating drops of the yolk sac/fix mixture. Slides were then placed on the rim of a beaker of boiling water to steam for 30 seconds. After drying, slides were washed in 3:1 fix solution and allowed to dry at room temperature overnight.

2.5.3. Hybridization of FISH slides

Embryonic yolk sacs and tail blood samples were both used for FISH utilizing molecular probes created from the BAC clones 401C2 and 433G17 (MOORE *et al.* 1999). Fixed slides as prepared above were placed into coplin jars containing pre-warmed 2X sodium chloride and sodium citrate solution (SSC) and were incubated at 37ºC for 30 minutes. After incubation, the slides were taken through a series of ethanol washes of 70%, 85%, and 100% ethanol for two minutes each. A 10:1 Denhyb (Insitus Biotechnologies, Albuquerque, NM) BAC probe solution was mixed and let warm in a 37ºC water bath. The backs of the slides were quickly dried and warmed to 37ºC on a dry bath in a darkened room for 3 minutes. Then 7 μl of the pre-warmed denhyb:probe mixture was added to the slide, covered with an 18x18 mm glass cover slip, and sealed with rubber cement. The slide was transferred quickly to a pre-warmed 85ºC dry bath for 5 minutes, after which the slides were rapidly placed into a 37ºC humidified chamber overnight.

2.5.4. Visualization of FISH slides

The following day the rubber cement was removed in a darkened room and the slides were placed into a coplin jar containing 2X SSC in a 68ºC water bath. The slides were incubated at 68ºC for 5 minutes and then transferred to a new coplin jar with room temperature 2X SSC in which they were incubated for 7 minutes in the dark. During this process, 4',6-diamidino-2-phenylindole (DAPI) (Chemicon International, Temecula, CA) was allowed to come to room temperature. Following the incubation, slides were mounted with 8 µl of Antifade/DAPI, and covered with a 22x22 mm glass cover slip. The slides were immediately viewed on a Nikon Eclipse 80i microscope with fluorescent capability and scored by the number of cells that could be counted to have the Ts65Dn marker chromosome.

2.6. Cell culture

2.6.1. Dissection of the E10.5 embryo

Pregnant female mice were sacrificed ten and a half days after a vaginal plug had been visualized. The mice were then anesthetized with isoflurane (Webster Veterinary Supply, Inc.) and euthanized by cervical dislocation. Embryos were dissected out and placed in Phosphate Buffered Saline (PBS) (Mediatech, Herndon, VA) on ice to induce hypothermia. Embryos were dissected in PBS, and yolk sacs were removed for later genotyping. Somites were counted and recorded and embryos were photographed with a Nikon Digital Sight Camera at 3X magnification.

Using 30G needles, the BA1 was removed from the embryo and neural tube sections were removed between the midbrain/hindbrain demarcation and the otic vesicle. Both tissues were dissected into approximately eight smaller portions and placed in 85μL of MCDM Medium (CHAI 1998) on 96-well human fibronectin coated culture plates (BD Sciences, Bedford, MA). Cells were allowed to proliferate medium was replaced approximately every 3 days or as needed.

2.6.2. LacZ staining for whole embryos

The remaining portions of embryo from the dissection (head and tail) were placed in 1.5 mL Eppendorf tubes with 0.5 mL PBS. When ready to stain, the PBS was removed using a transfer pipette and replaced with X-gal solution (25 μL of 10% X-gal in X-gal buffer (0.02 g potassium ferrocyanide, 0.016 g potassium ferricyanide, 9.6 mL wash buffer (0.025% $MgCl_2$,1% Nonidet-P40 (IPEGAL))). Embryos are then incubated in a 37°C water bath for 2 hours for until some turn blue.

2.6.3. MCDM medium

To a 15 mL conical vial, 8.7 mL low-glucose DMEM (Gibco, Grand Island, NY) was added, along with 10% Chick Embryo Extract (CEE) (SLI, West Sussex, UK), 20 ng/mL bFGF, 1% N2, 2% B27 (Gibco, Grand Island, NY), 50 μM 2-mercaptoethanol, 35 ng/mL retinoic acid (Sigma, St. Louis, MO), and 25 u/mL Penn/Strep. The solution was filtered through a 22 μm filter and a 20 mL syringe into another 15 mL conical vial (ZHAO *et al.* 2006).

2.6.4. Fibronectin coating coverslips

A 22x22 glass coverslip was outlined using PapPen (RPI Corp) and placed into a plastic petri dish. The coverslip was flooded with Poly-D-Lysine (100 µg/mL in HEPES) and incubated at 37°C for 30 minutes. After the incubation the coverslips were washed twice with HEPES and flooded again with human fibronectin (BD Sciences, Bedford, MA) at a concentration of 5 $\mu g/cm^2$ of area to be covered. The fibronectin/HEPES solution was incubated on the coverslips at 37°C for 30 minutes. Following the incubation, after the coverslips were rinsed twice with HEPES, they were rinsed with culture medium before cells suspended in medium were added to them and allowed to attach to the fibronectin at 37°C overnight.

2.6.5. Proliferation assay

A 10µM 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) solution was made in warmed MCDM. NC cells grown on glass coverslips at 80% confluency were incubated with the EdU solution for 30 minutes in a 37°C incubator. Following the incubation, the EdU solution was removed and replaced by 4% paraformaldehyde in PBS to incubate for 15 minutes at room temperature. Following this incubation, the paraformaldehyde was removed and the coverslip was washed with 3% BSA in PBS. After the wash solution was removed, the NC were incubated with 0.5% Triton X-100 in PBS at room temperature for 20 minutes. No more than 15 minutes before use, the Click-iT cocktail (utilizing the Alexa Fluor 594) was prepared according to the manufacturer's instructions (depending on number of coverslips used). The Triton solution was removed and the NC

were washed again in the BSA solution. Coverslips were incubated with the Click-iT cocktail for 30 minutes at room temperature, protected by the light. After the incubation, coverslips were again washed with the BSA solution, and 7 µL DAPI (Chemicon International, Temecula, CA) was added to the coverslip. The coverslip was then inverted onto a glass slide and sealed into place using clear fingernail polish. Assay was visualized a Nikon Eclipse 80i microscope with fluorescent capability and scored as the percentage of cells fluorescing red of total cells.

2.6.6. Senescence assay

The following solutions were made:

- *Fix solution*: 2% formaldehyde, 0.2% gluteraldehyde in PBS
- *0.2 M Citric Acid/$NaPO_4$ Buffer at pH 6.0*: 36.85 mL of 100 mM citric acid solution and 63.15 mL of 100 mM Na phosphate solution
- *Staining solution*: 40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ $3H_2O$, 5 mM $K_3[Fe(CN)_6]$, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/mL X-gal in distilled water.

NC cells were grown to subconfluency on fibronectin coated coverslips and washed with PBS. Coverslips were incubated with Fix Solution for 5 minutes at room temperature and then washed twice with PBS. Coverslips were then incubated with Staining Solution overnight at in a non-CO_2 incubator at 37°C. The following day, cells were washed twice with PBS and once with methanol and allowed to air dry. Coverslips were then inverted and mounted onto glass slides with clear fingernail polish.

2.6.7. Fluorescein digalactoside staining

LacZ positive cells must be used for the fluorescein-di-beta-D-galactopyranoside (FDG) (Molecular Probes, Eugene, OR) stain to work properly. Negative lacZ cells were used as a control. Confluent NC cells on fibronectin plates were dislodged using 1X trypsin for 4 minutes. Low glucose DMEM (Gibco, Grand Island, NY) was used to stop the reaction and cells were triterated to release them from the plate. After removal of the cells into 1.5 mL tubes, they were centrifuged at 4°C and 10,000 RPM for 10 minutes. Supernatant was removed and cells were resuspended in 30 μL DMEM. From this solution, 25 μL were transferred to a 4 mL glass FACS tube and were incubated in a 37°C water bath for 5 minutes. While incubating, a 1:1 FDG solution was made using 2mM FDG (diluted from 20 mM in water) and DMEM. The cells were then incubated with the FDG solution in a 37°C water bath for 1 minute. The samples were diluted with 450 μL of ice cold DMEM and then put on ice for 30-60 minutes. After FACS sorting into NC and non-NC cells, they were transferred to 1.5 mL tubes and spun down at 10,000 RPM and 4°C for 10 minutes, and seeded onto fibronectin coated plates.

2.6.8. Immunocytochemistry of NC cells

NC cells were cultured until approximately 75% confluent on fibronectin coated coverslips. MCDM media was removed and the cells were washed twice with PBS (Cellgro, Herndon, VA). The coverslips were incubated with 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature. After the incubation, the coverslips were washed three times with PBS. Coverslips were then incubated with 0.5% Triton

(Promega, Madison, WI) in PBS for 10 minutes at room temperature followed by three washes with PBS. The coverslips were incubated in 10% donkey serum (Gibco, Grand Rapids, NY) in PBS for 30 minutes at room temperature and washed 3 times for 5 minutes each. The polyclonal primary antibody, HNK1 (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal primary antibody, AP-2α (AbCam, Cambridge, MA) were both used at a 1:250 dilution in PBS. Coverslips were incubated with either antibody for 1 hour at room temperature and washed three times for 5 minutes each. In a dark room, the secondary antibody at a 1:500 dilution in PBS was added to the coverslips and was allowed to incubate for 30 minutes at room temperature followed by three washes of 5 minutes with PBS. DAPI was added to each coverslip, which was then inverted, put on a glass slide and mounted with clear fingernail polish.

CHAPTER 3. RESULTS

3.1. General results of phenotypic occurrences

Of the 137 infants who were recorded to have visited during the specified time frame, only 119 had sufficient data to be included in the final pool of subjects. Mean birth weight, length, head circumference, and Apgar scores are listed in Table 9, in addition to the breakdown of ethnicity of all infants included in the study as compared to the Riley Neonate Unit.

To assess the data as a whole, all information recorded regarding a specific organ system were grouped together, resulting in a general presentation of anomalies within a specific area. Overall, 80% of infants had a recorded cardiac anomaly at some time during the first six months and 60% had either a structural or functional gastrointestinal (GI) abnormality. We believe the high incidence of cardiac defects as compared to the literature can be attributed to the nature of the Down Syndrome Clinic at Riley Hospital as a referral clinic. Additionally, we are limited by the method of obtaining cardiac anomaly records, as most are from echocardiogram results from the first or second day of life. This can result in the recording of anomalies that may have spontaneously closed. Nearly half of infants had aspiration or penetration recorded as a result of a feeding evaluation, but this does not include those with any feeding difficulties not assessed specifically by videofluoroscopic feeds. Additionally, approximately a third of infants

had either airway, including obstructive sleep apnea and upper airway occlusion, or auditory abnormalities in the first six months of life. Several infants presented with abnormal endocrine functions (Figure 1).Feeding strategy correlates with the presence of cardiac and GI abnormalities.

3.2. Feeding results

3.2.1. Feeding strategy correlates with the presence of cardiac and GI abnormalities

Of the 119 infants included in this study, 115 had data regarding feeding recorded from a clinic visit during the first six months of life. Data collected included feeding route, type of liquid feed, and any alteration to the liquid. To investigate the feeding strategy for infants visiting the clinic, the route of feeds, per oral or tube, were recorded and grouped in monthly intervals to track any trends seen in strategy in the first six months (Figure 2). Oral feeds were defined as ingestion by breast or bottle and tube feeds are by nasogastric or gastronomy tube. It was also of interest to investigate the type of oral feeding occurring, be it breastfed or bottle-fed alone, or a combination of the two strategies (Figure 3). In the neonate period (<30 days of life), when most infants are seen for the first time in the clinic, bottle feeds and tube feeds occur more often, but infants are still being breastfed. As time progresses, we see very few infants being breastfed, and bottle and tube feeds are still the most common.

In order to examine the modification in feeding strategy over time, a subpopulation of infants with at least two clinic visits (83 of 115) were more closely

investigated. Using this approach, a better understanding of any changes in the method of feeding and why the alteration may have occurred could be gained. Again, oral and tube feeds were compared to find that of the 83 infants with multiple visits, 64% were feeding orally and 36% were feeding by a tube in their earliest visit, most often during the neonate period. However, when the last visit these infants had in the first six months was examined, the percentages of oral and tube feeds were exactly the same (Figure 4). These results may be deceiving and seem to imply that the infants seen multiple times in the clinic do not experience any modification in their feeding strategy. To investigate this more closely, the modification of feeding route between the earliest and latest visit was examined. Infants were grouped into one of four categories based on their initial and final feeding strategy recorded: early oral feeding to late oral feeding (O→O), early oral feeds to late tube feeds (O→T), early tube feeds to late oral feeds (T→O), and those that fed by tube both early and late (T→T). It was found that 49% of infants began feeding orally and were able to continue an oral feed, and 22% of infants that began tube feeds were feeding by a tube in their last visit. However, 15% of infants had begun feeding orally and were required to switch to tube feeds, and 15% were moved from a tube feed to feeding orally in the first six months (Figure 5). It can also be seen that the number of infants breastfeeding decreases as those being fed by bottle increases when feeding route is examined over time for those with two visits (Figure 6).

To further examine the relationship between the alteration of feeding route in the first six months with other abnormalities, correlations with cardiac and GI abnormalities were investigated. Of these infants with at least two clinic visits, those with severe cardiac abnormalities were just as likely to continue feeding orally as those without a

cardiac anomaly. However, infants with a severe cardiac abnormality were more likely to have been tube fed through the entire 6 months as compared to infants without a severe cardiac defect (Figure 7). Infants with severe GI abnormalities were much more likely to visit the clinic twice in the first six months, with those feeding by tube the most common. Those without a GI abnormality and had been seen more than once appear to be able to feed orally from the beginning by a large margin when compared to those with a severe GI abnormality (Figure 8).

3.2.2. Muscle tone as evaluated by DQ may indicate best feeding strategy

To evaluate the role of muscle tone on feeding strategy, sixty-four infants had evaluations of their DQ during clinic visits. Low muscle tone in infants and adults with Down syndrome has been widely documented and the gross developmental quotient (DQ) was used as a quantitative means of measurement of tone. From these 64 infants, the overall average estimate of muscle tone was a DQ of 55. This average remained approximately equal, even if the DQ is plotted across time in accordance to when the DQ was taken, indicating the time when the evaluation was recorded did not seem to play a role in the accuracy of assessment. Surprisingly, there appears to be no difference in the mean DQ of infants feeding orally as compared to those ever fed by tube in the first six months. However, when an alteration of feeding strategy in the first six months was considered, those who had remained on either oral or tube feeds had an average DQ of 57, but those that had switched feeding routes from either oral to tube or tube to oral had average DQs of 35 and 63, respectively (Figure 9).

As the developmental quotient was further investigated, it was found that infants with severe cardiac defects did not have lower muscle tone as compared to those with no cardiac abnormality. The interaction between DQ and the presence or absence of a cardiac abnormality does not appear to determine feeding strategies in infants with DS. Alternately, infants with severe GI abnormalities were more likely to have a DQ below 50. Additionally, those with a severe GI abnormality and a low DQ were more likely to need to switch from oral feeds to tube feeds, indicating the interaction between GI abnormalities and DQ may assist in establishing the most beneficial feeding strategy (Figure 10).

3.2.3. Video evaluations can provide a better understanding of feeding complications

Sixty-five infants with noted feeding difficulties by either parents or clinical observation were evaluated by pulse oximetry swallows or barium videofluoroscopic studies. Only 11 infants were evaluated by barium swallows while 53 infants underwent oximetry swallows. One chart did not have the method of investigation recorded, but data from this record was still used in analysis because diagnosis of penetration or aspiration could not be made without videofluoroscopic evaluation. From all evaluations, we found that 69% of infants had penetration of thin or thickened liquid feeds. Penetration of feeds indicates the passage of liquid or food into the airway. Additionally, 55% of these infants aspirate their feeds either on thin or thickened liquids.

To investigate the severity of results from feeding evaluations, incidences of aspiration or penetration alone were separated from incidences of aspiration and

penetration occurring together, with or without additional complications. Additional complications include oral-motor dysfunction and oxygen desaturation during feeds, and are detailed in Table 10. For simplicity, in graphs and tables, these additional complications noted during evaluations are simply designated "other." Despite the innocuous title, half of the infants experience pharyngeal pooling of the liquid feed, and high percentages of the infants have drops in blood oxygen and display difficulties in oral-motor function. Aspiration occurred as an isolated event in 8 infants while penetration occurred alone in 17 infants. Penetration and aspiration were found together in 27 infants, indicating that 42% of infants experience not only the penetration of food into the airway, but it passes into the trachea, creating considerable discomfort and feeding difficulty. Other complications presented in 80% of infants, and in nearly all infants with both penetration and aspiration, some additional complication occurred (Figure 11).

3.3. Comorbidities of cardiac, GI, feeding, and airway abnormalities

After examining the data in general terms, we investigated the comorbidity of anomalies in two systems. While 7% of all infants had a severe cardiac defect alone, nearly three quarters of all cardiac abnormalities were found to be indicative of GI abnormalities or feeding difficulties (Table 11). GI abnormalities also seem to be predictive of feeding complications. From this information, we hope to assist medical professionals in their health care of infants with DS by understanding the correlations between those phenotypes that are often recognized early, such as cardiac and GI

anomalies, with those that often go unnoticed for longer periods of time, such as feeding or airway difficulties. In order to understand our results further, we examined each system with greater detail.

3.3.1. Severe cardiac abnormalities appear to correlate with GI, feeding, airway, and auditory complications

Cardiac defects were defined as the presence of an atrial septal defect (ASD), ventricular septal defect (VSD), atrioventricular canal defect (AVCD) or patent ductus arteriosus (PDA) in the first six months. These include those that spontaneously repaired, were surgically repaired, or were still present after 6 months. Ninety-five infants were recorded to have at least one of these abnormalities, and an additional seven had a patent foramen ovule (PFO) alone but were excluded from all analyses due to the normality of this diagnosis by echocardiogram on the first day of life. ASDs were most common in the population either alone or in combination with another anomaly, with PDAs occurring second most often, again, either alone or in combination (Figure 12). ASDs alone occurred in 19% of infants and another 15% of the population experienced only a PDA. However, both an ASD and PDA occurred together in an additional 21% of 95 infants recorded to have a cardiac defect, indicating either an ASD or PDA, alone or in combination with one another, occur in more than half of all infants with DS.

In order to further investigate cardiac anomalies in infants with DS, we examined those with a severe cardiac defect as a more specific population to compare with those never recorded to have a cardiac abnormality in the first six months. Severe cardiac defects were defined as those that required either surgical repair or treatment by a diuretic

or Digoxin. Twenty-eight infants were found to have a severe cardiac anomaly, with nine needing surgical repair at an average age of 3.5 months. Only five infants were given Digoxin, but 27 were on some type of diuretic. When compared to the 24 infants without any cardiac defect, those with a severe cardiac abnormality are more likely to have GI abnormalities (p=0.099, Fisher's exact test; odds ratio=2.5; n=52) as well as obstructive sleep apnea (p=0.085, n=21) and may be associated with feeding (p=0.11, χ^2 goodness of fit test, n=31) (Figure 13). This data could provide reason to screen all infants with documented cardiac abnormalities for other difficulties, including videofluoroscopic feeds and polysonograms in the first six months.

3.3.2. Severe cardiac, GI, and airway abnormalities appear to correlate with videofluoroscopic data

Severe cardiac defects were defined as those requiring surgical repair or medication. Nearly three quarters of infants with a severe cardiac defect had some type of feeding difficulty, either penetration, aspiration, or both, as determined by a feeding evaluation. Of the 25 infants with both penetration, aspiration, and an additional complication, 10 individuals had a severe cardiac defect while only 3 infants had no cardiac anomaly. This data appear to indicate that the presence of a severe cardiac anomaly correlates with feeding problems in infants with DS.

GI anomalies that required repair or medication were classified as severe. Of the 45 infants with a severe GI abnormality, 90% had some type of feeding difficulty compared to only 36% of infants without GI problems (p<0.001, χ^2, odds ratio=1.7, n=45) Severe GI structural anomalies occurred in only 3 infants with feeding evaluations,

and all these presented with both penetration and aspiration in addition to another problem noted during evaluation. Another manner of examining the same results reveals more than half of the infants that had been evaluated for feeding had a severe functional GI anomaly as compared to only a quarter without a GI abnormality. Like cardiac defects, it appears as if severe GI anomalies closely correlate with feeding difficulties.

Infants with feeding difficulties may often present with a number of airway problems, including sleep apnea or the need for supplemental oxygen. Aspiration, penetration, and other feeding difficulties occurred together in 29% of infants with obstructive sleep apnea (OSA) ($p=0.17$, χ^2, $n=34$) and in 38% of infants with upper airway occlusion (UAO) ($p<0.001$, χ^2, $n=34$). Additionally, it was noted that 39% on infants with penetration, aspiration, and other difficulties while feeding required O_2 supplementation at some time during the first six months. Together, these data may indicate a close relationship with feeding complications and airway anomalies.

The mean DQ for the 40 infants with feeding evaluations and a documented DQ was 54, nearly the same as the mean DQ of 57 for the entire population. Of the infants with a feeding evaluation, 16 had DQs less than 50 and 22 infants had DQs greater than 50. Interestingly, infants with other complications noted during the evaluation were more likely to have DQs above 50, but penetration or aspiration alone or together showed no difference in DQ. This appears to indicate that low muscle tone as described by DQ may not be a predictor of results from a feeding evaluation, but it should not be seen as negative evidence to the need for an evaluation in the first six months for infants with DS.

To combine the data from feeding evaluations with alterations made during clinic visits, the correlation between altered feeding strategy and feeding difficulties was examined. Of the infants with a modification in feeding and at least two visits in the clinic, 96% had undergone feeding evaluations. It was found that infants without feeding difficulties were most often able to remain on oral feeds from the first visit. However, those with difficulties were either on tube feeds over the course of six months, or experienced a modification in strategy (Figure 14). In addition, when investigating the relatedness of either a cardiac or GI anomaly with the feeding route for those with a feeding evaluation, it was found that the presence of either abnormality closely mirrored the pattern for feeding strategy. This seems to imply that a cardiac or GI abnormality could correlate with aspiration, penetration, or other feeding problems (Table 12). Therefore, the presence of a cardiac anomaly, GI abnormality, airway complication or a low measure of hypotonia could assist in the determination of the recommended feeding methodology for infants with DS.

3.3.3. Gastrointestinal abnormalities correlate with feeding and airway difficulties

Gastrointestinal abnormalities include both structural defects such as Hirschsprung disease and duodenal atresia, and functional difficulties including gastroesophogeal reflux (GER) and constipation. A listing of all structural anomalies recorded can be found in Table 13. In addition to a large proportion of infants with functional difficulties due to GER and constipation, there was one count each of delayed gastric emptying and cow milk protein intolerance in separate individuals. GI abnormalities were found in 60% of all infants, and of these, both GER and constipation

were twice as likely to occur than a structural defect, which were found in only a quarter of the individuals with recorded GI abnormalities (Figure 15). Hirschsprung disease and duodenal atresia were each found in approximately 5% of all infants, and other abnormalities include an imperforate anus, the most common cause for surgical repair. In total, 8 infants had some type of surgical repair in the first six months of a GI structural defect. Additionally, 50 of the 71 infants with a GI abnormality were on some type of medication. These include proton pump inhibitors, H2 blockers, prokinetics, Senna, or polyethylene glycol (PEG, or Miralax). Of those on medications, 90% also had a functional difficulty (GER or constipation), and more than half were on multiple types. Understanding the widespread occurrence of functional GI difficulties infants with DS experience should provide reason to be aware of the likeliness that an infant may be in distress, with or without a parents' notice.

To examine the correlation of GI abnormalities with other abnormalities, we investigated the difference between infants with a GI abnormality and those without. When the two populations were compared as they correlated with other abnormalities, we found that GI abnormalities highly correlated with feeding difficulties, where 65% of those with a GI abnormality have a feeding complication that was identified during a feeding evaluation, and only 31% of infants without a GI abnormality had a feeding difficulty ($p<0.001$, χ^2, n=61). Likewise, abnormal auditory screens were more common in infants with a GI abnormality as compared to those without (p=0.18, Fisher's, odds ratio = 1.9, n=94). Airway difficulties are also more common in those with a GI abnormality (p=0.09, χ^2, n=43) (Figure 16). These results appear to indicate the correlation of GI abnormalities with feeding, airway, and auditory anomalies, which

could suggest that once an infant is screened for and found to have GI difficulties, the next step should be a videofluoroscopic feed and a referral to an otolaryngologist.

3.3.4. Airway anomalies found in all infants with PSGs may imply some cases go undiagnosed

We have defined airway abnormalities as diagnosed by obstructive sleep apnea (OSA) and upper airway occlusion (UAO) by polysonograms (PSGs). Overall, 43 PSGs were carried out, 38 of which were nap studies and 3 were carried out overnight. The mean age at which PSGs were completed was 1.5 months. There were 25 counts of OSA documented, four of which were classified at mild, 6 were moderate, and 6 were considered severe cases. Additionally, 39 cases of UAO were found. Most importantly, all 43 infants were documented to have at least one of these airway difficulties during sleep. Mean apnea and hypopnea occurrences and indices can be found in Table 14. Most often PSGs are the result of parental concern of the infant snoring during sleep or wheezing during the day. However, infants with DS may suffer from airway complications that can perhaps go unnoticed or do not appear severe enough to mention to a medical professional during an exam. In finding that all infants suspected of breathing difficulties were found to experience either OSA or UAO, and some infants may not display symptoms or go unnoticed, it could be beneficial to screen all infants for airway complications in the first six months of life, especially those with a cardiac or GI abnormality.

3.4. Cell culture results

The use of an *in vitro* model can be beneficial to examine specific qualities of a larger system. To develop this model, the BA1 and a segment of the neural tube between the midbrain/hindbrain demarcation and the otic vesicle were removed from E10.5 embryos. The pieces of tissue were placed on fibronectin-coated plates and the NC migrated away from within them. Culturing of the NC allowed for the possibility of future experiments to help determine the cause of the small BA1 at E9.5. In total, approximately 180 embryos were removed from mothers of which only a small portion were able to passage to adequate numbers. First, the tissue sections were simply placed on the plate and allowed to grow, being fed as needed. At the second day, approximately 85% looked promising, with a relatively large number of stellate cells surrounding the tissue. Passaging cells from a 96-well plate to a 48-well plate generally created a further 20% loss in cells, of which only 85% appeared to be growing at the second day after passage. There was an approximate 85% success rate when transferring cells to a 24-well plate, where 75% looked stellate two days later. Together this represents a 75% loss in cells.

In an attempt to increase the number of samples that would continue to be successful after several passages, numerous methodologies were investigated to produce the most reliable results. After several attempts to ensure the attachment of the NC to the culture dishes, the most successful method includes shredding of the tissue. Both the paired BA1 structure and the neural tube explants were torn into approximately eight smaller sections to be distributed onto the fibronectin-coated well already containing culture medium. This allowed several portions of tissue to attach to the well and the

successful migration of more NC away from the tissue. Additionally, we have found that the passage of NC is most successful when culture medium is replaced the day before passage, and the passage occurs when the cells are approximately 75% confluent. If they are allowed to proliferate past this point, the cells begin to die despite an initial period that may appear successful. It was also found that the centrifugation of trypsinized NC in order to remove the cells from any remaining trypsin resulted more often than not in a loss of many NC. This creates difficulty as we not only begin with a relatively small number of cells, but the NC are more likely to reattach to new culture wells after passaging if the cell density is high. Therefore, passaging at 75% confluency and removal of the trypsin after a shorter incubation period to allow the direct transfer of NC between wells (as further described in the Materials and Methods) provides the most successful method of culturing NC. Using this method, approximately 94% of samples had significant numbers of stellate cells. The success rate for passaging cells remained approximately the same, at an estimated 80% for 96 to 24-well plates and 85% from 48 to 24-well plates. Also, approximately 85% of samples looked good two days after passaging. Using this method, there was only a 55% loss of samples, greatly increasing the number of cells available for assays.

After increasing the success culturing of the samples, it was of interest to examine the percentage of NC in the sample, as the mesenchyme is also plated. To do so, lacZ positive cells were counterstained using FDG and sorted using a FACS analyzer. From seven samples that were sorted, an average of 70% were FDG positive, or identified to be NC. The BA1s had approximately 81% NC, while the neural tube samples were comprised of approximately 56% NC. Additionally, the earliest sort that was performed

five days after dissection from a BA1 sample had 99% of the cells to be NC while a BA1 sort 19 days after dissection revealed only 67% NC. This could indicate that the NC cells are not proliferating to the same extent as the mesenchymal cells surrounding them. Brief immunocytochemistry experiments were performed to label the NC specifically. It appears that approximately 60% of cultured samples were NC 14 days after dissection.

To investigate the role of proliferation and senescence in the NC, both the BA1 neural tube segments were again removed from E10.5 embryos. Beginning these experiments it was thought best to allow each culture well to become confluent with NC before the passage of cells. However, we have found that the passage of NC is most successful when culture medium is replaced the day before passage, and the passage occurs when the cells are approximately 75% confluent. If they are allowed to proliferate past this point, the cells begin to die despite an initial period that may appear successful. It was also found that the centrifugation of trypsinized NC in order to remove the cells from any remaining trypsin resulted more often than not in a loss of many NC. This creates difficulty as we not only begin with a relatively small number of cells, but the NC are more likely to reattach to new culture wells after passaging if the cell density is high. Therefore, passaging at 75% confluency and removal of the trypsin after a shorter incubation period to allow the direct transfer of NC between wells (as further described in the Materials and Methods) provides the most successful method of culturing NC.

To investigate proliferation and senescence directly, two assays were utilized, as described in the Materials and Methods, on cultured NC. The proliferation assay appears to give more fruitful results when the NC are allowed to proliferate until near confluency, whether on fibronectin-coated coverslips or culture slides. Preliminary results using only

one sample from both a trisomic and euploid embryo indicates no difference in proliferation, approximately 45% of total NC, one day after tissue extraction (Figure 17). To measure senescence, the detection of 'senescence-induced β-galactosidose' activity (SA-βgal), however, must be carried out on subconfluent cultures, as confluency has been reported to induce SA-βgal activity. At present, we have only tested this assay on senescent BJ foreskin fibroblasts graciously donated by Dr. Herbert's lab (HERBERT *et al.* 2003), but have shown that the SA-βgal activity is detectable in these cells and are ready for use in future projects (Figure 18).

CHAPTER 4. DISCUSSION

4.1. Comorbidities between organ systems demonstrate the close relationship between phenotypes

Individuals with Down syndrome present with a wide array of clinical phenotypes including, but not exclusive to, cognitive impairment, craniofacial dysmorphology, cardiac defects, gastrointestinal abnormalities, airway complications, feeding difficulties, and auditory impairments (EPSTEIN 2001b; ROIZEN 2003b; VAN CLEVE 2006). The incidences of these phenotypes in individuals with DS have been reported on numerous occasions, and studies have been conducted to investigate the genetic foundations behind them (ANTONARAKIS *et al.* 2004; EPSTEIN 2006; KORENBERG *et al.* 1990b; PATTERSON and COSTA 2005; WEIJERMAN *et al.* 2008). However, most studies do take into account the benefit that could be reaped from understanding the relationship between these phenotypes. By understanding the relationship of co-occurrences of clinical traits, one can provide medical professionals with the tools to better assist those with DS by giving them a basis of what to look for in an individual. Discerning comorbidities for these phenotypes has therefore been the aim of this study, in order to provide the best healthcare for individuals with DS based on an anticipation of needs.

Here we have taken data regarding the major organ systems affected in individuals with DS including cardiac, GI, feeding, airway, auditory, and endocrine.

Cardiac abnormalities are reported to occur in 44% of individuals with DS, and here we have recorded 80% of infants were found to have some type of CHD (FREEMAN *et al.* 2008). Data from the NDSP provides the most comprehensive records regarding abnormalities, but can be taken from any time point in an individual's life. We are limited by our ability to record from the first 6 months of life, in investigating infants alone and thereby an IRB exemption, and in regards to cardiac incidences, ours will appear high because of our inability to discern spontaneously closed abnormalities and artifacts remaining from the development of fetal circulation. Because EKGs are most often recorded on the first day of life if an infant is suspected of DS, artifacts of fetal circulation such as PFOs and ASD vs PDAs will be recorded. However, a much better understanding of the infant's heart could be discerned from EKGs after the first week. To counteract this restriction, severe cardiac abnormalities were investigated in order to gain a better understanding of the comorbidity of cardiac abnormalities with other anomalies. Freeman *et al.* reports 20% of individuals with a cardiac abnormality to present with another anomaly, but here, we demonstrate that nearly three quarters of infants with severe cardiac abnormalities have GI or feeding complications, and nearly half also have airway or auditory difficulties (FREEMAN *et al.* 2008). In the present study, we demonstrate not only the underestimation of how often abnormalities co-occur in individuals with DS, or even how few times the comorbidities are investigated, but more importantly, that infants with severe cardiac abnormalities should be further screened for all other complications, especially those not often looked for in the first year of life such as feeding and airway complications.

Studies regarding GI abnormalities most often focus on structural defects rather than functional complications that result. Again, the NDSP has the most comprehensive reports and has found that GI abnormalities occur in approximately 7% of the DS population. Here we find 15% of infants have structural GI defects. However, Freeman *et al.* does not report on the functional difficulties individuals with DS experience. Perhaps GER and constipation are considered "normal" for an infant with DS, which results in overlooking these phenotypes (DAVIDSON 2008). Yet we found that together, infants with structural and functional GI abnormalities are much more likely to have feeding, airway, and auditory complications. And even the functional GI anomalies alone should be considered reason to investigate the individual further, as GER and constipation can be indicative of overall larger difficulties such as esophageal dysfunction (HILLEMEIER *et al.* 1982). This again demonstrates the need to screen early and effectively for further difficulties beyond those stressed by the healthcare guidelines.

4.2. Comorbidites between cardiac and GI abnormalities with feeding complications demonstrate the need for early screening

As the comorbidities of abnormalities within entire organ systems were examined, the relationship between abnormalities and feeding complications was investigated. Overall, infants with DS experience feeding difficulties as a result of not only structural differences in the craniofacial skeleton, but functional anomalies such as oral-motor difficulties, decreased suckle ability, weak lip seal, decreased suck/swallow reflex, poor lip coordination, and difficulties maintaining suckling rhythm (COOPER-BROWN *et al.*

2008; FAULKS *et al.* 2008; HENNEQUIN *et al.* 1999; LAZENBY 2008; MIZUNO and UEDA 2001). Over time it appears that the frequency of breastfeeding decreases as mothers opt for bottle or tube feeds instead, despite the noted benefits to breastmilk (AUMONIER and CUNNINGHAM 1983; BIDDER *et al.* 1975; FIELD *et al.* 2003). Many mothers of infants with DS express their frustration as their infant has difficulty breastfeeding, but perhaps instead of switching to another feeding route, medical professionals could offer more therapies on how to stimulate the suckle ability while breastfeeding to encourage proper suck/swallow reflexes and improve overall oral musculature (ARVEDSON 2008; FIELD *et al.* 2003). However, the question remains how to investigate not only the difficulties experienced by individuals with DS while feeding, but the correct course of action. Early therapies to improve reflexes may not be enough to counteract aspiration of liquid feeding.

To examine feeding routes and their alterations over time, we investigated not only how the infants were feeding, but with what did strategy correlate. There is very little to be said as to how an infant should feed when comparing those with severe cardiac abnormalities and those without. Aumonier and Cunningham reported approximately 56% of individuals with DS had both cardiac lesions and difficulties breastfeeding, and our results indicate a near significance with severe cardiac defects with feeding complications. However, the presence of a GI abnormality would be a much better predictor of optimal feeding strategy, as those without GI abnormalities appear to feed orally without problem in the first six months. The presence of a GI abnormality appears to indicate tube feeding, at least for a time, could be beneficial for the infant. Additionally, developmental quotient (DQ) as a measure of muscle tone, appears to be a

good indicator of feeding strategy, as infants with lower than normal DQs are more likely to require tube feeds in the first six months. Together, while the presence of a GI abnormality would indicate the need for a feeding evaluation to investigate the complications that could exist, it can aide in decisions regarding feeding strategy in the meantime.

In order to recommend the best feeding route for infants with DS, these results suggest videofluoroscopic studies should be the new gold standard, much like EKGs are done for infants with DS on the first day of life. Using these types of studies, we may begin to understand the complications faced by the individual (ARVEDSON 2008; DEMATTEO *et al.* 2005; FAULKS *et al.* 2008; LAZENBY 2008; ROIZEN 2003a). These studies have demonstrated the benefits of feeding evaluations to accurately understand the complications experienced by the individual. With a feeding evaluation, the degree of severity can be determined with more accuracy than a simple bedside observation, as it has been demonstrated some infants can be silent aspirators (SPENDER *et al.* 1996). Many individuals with DS do not express pain in the same manner as individuals without DS, and may not display any discomfort (LAZENBY 2008). Often, complications can be relieved by the alteration of the feeding strategy, such as increasing the thickness of the liquid feeds or using a different rubber nipple (ARVEDSON 2008; COOPER-BROWN *et al.* 2008; FAULKS *et al.* 2008). Simple changes such as these improve the quality of life for the infant and parents as the infant may now have the opportunity to feed painlessly, receive proper nutrition, and develop proper attitudes about eating (RANWEILER 2009; ROIZEN 2003a). These can relieve possible complications later in life by improving nutrition as poor nutrition habits can become habitual. They can affect the individual

socially by impeding possible integration, and the possibility that the individual will shy away from foods he or she does not like based on difficulties while feeding (FIELD *et al.* 2003; LAZENBY 2008).

In conclusion, our study of the comorbidity between organ systems demonstrates the close relationship between severe cardiac and GI abnormalities with feeding and airway difficulties. Current DS healthcare guidelines do not recommend the assessment of feeding or airway complications in the first year of life. However, not only will many individuals with DS continue to have difficulties that will go unnoticed for long periods of time, but aggressive and effective screening has the potential to improve the quality of the infant's life.

4.3. Discussion of NC cell culture

The current picture regarding the NC of both trisomic and euploid embryos at E9.5 and E10.5 is lacking. While the quantitative studies have been done to demonstrate the smaller BA1 with fewer NC at E9.5, the understanding of what is specifically occurring within the NC has not yet been quantified. An *in vitro* model would be the best to investigate the NC at a finer level. Culturing the NC at these few times points has been a challenge, but the utilization of multiple tissue pieces allowed the NC to migrate from the mesenchyme and increase success rates 25%. In this modified method, using a shorter incubation time with the trypsin and carefully triterating the cells from the plate has also improved success. Additionally, the knowledge of passaging the NC before they become completely confluent has saved many lines. Without this, most cells

die before they can be used in further study. Together, these techniques allow for a useful *in vitro* system in which to study some specific mechanisms that may be affecting the NC.

Once the method of culturing NC had been improved, examining the percentage of NC present revealed approximately 70% of the sample to be NC, even two weeks after dissection. However, by FACS sorting 5 days after dissection, we find that an extremely high percentage of NC cells from the BA1 are present in comparison to the surrounding mesenchymal cells, indicating early cultures of BA1 tissue would be an ideal choice for further assays to examine the NC. Secondarily, the use of immunocytochemistry can also label NC when performing additional studies.

In order to further examine the difference in BA1 size at E9.5, the differences in proliferation were investigated. On the first day after dissection, there was no difference between trisomic and euploid in the number of cells proliferating, approximately 50% for both. This appears to indicate the proliferative capability of the NC is the same at E10.5, after the NC have settled within the BA1. With future studies, the same can be done with senescence, as SA-βgal activity is indicative of a cell becoming senescent. In the same manner, the percentage of cells undergoing cell-division arrest can be calculated and compared between trisomic and euploid. In all, both assays have begun to elucidate the characteristics of the NC both within the BA1 and those that have migrated from the neural tube.

CHAPTER 5. FUTURE WORK

5.1. Clinical future work

For future studies within our clinical data collection, we have planned to extend our current study to include infants that have visited the Down syndrome clinic through July 2010. With this new information, it will be possible to obtain more definitive cardiac data, where PFO vs ASD can be recorded separately from infants with only one or the other. This should aid in finding the true incidence of CHD as seen by the DS clinic, and will allow ourselves to more easily compare our results to those found by other researchers. On a larger scale, an increase in numbers of infants has the potential to greatly strengthen statistical data.

Additionally, while this researcher has thoroughly investigated the co-occurrences of feeding difficulties with other abnormalities, it would be of interest to better understand the role of feeding evaluations performed by pediatric specialists and their recommendations regarding feeding. One might investigate the interactions of feeding route, alterations to the feeding liquids, such as increased caloric or thickened qualities, and the results from feeding evaluations. Using this information, one might find that the combination of both feeding evaluations to diagnose any difficulties present, and any changes made to feeding liquids or route to correct these difficulties, allow for the

greatest success when feeding and therefore provide infants with the greatest chance to thrive.

5.2. Future studies in NC cell culture

As we understand the trisomic BA1 is not smaller at E9.25 when compared to euploid, but the BA1 from E9.5 trisomic embryos is comprised of significantly less NC than euploid embryos, one would be most interested to understand the mechanisms behind this difference. To do so, a time line for the NC could be investigated, where assays to determine proliferative and senescent qualities of the NC from E9.25, E9.5, and E10.5 embryos could be examined, both one day after dissection and after the NC have been cultured for a time. Understanding whether the NC are proliferating less or undergoing early senescence at a particular time point could allow for therapies to increase the NC and perhaps restore the BA1 of trisomic embryos to a normal size. Lastly, to further elucidate the possible means by which the NC are in smaller quantities in the trisomic BA1, qPCR could be performed to understand the differences on an RNA level.

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TABLES

Table 1: Template of birth information recorded. Recorded from infants with DS in the first six months. Bold letters indicate single letter codes used for simplicity.

Birth Information	
	Gestational age (wk)
	Birth weight (g)
	Birth length (cm)
	Occipitofrontal circumference (cm)
	APGAR 1
	APGAR 5
	APGAR 10
Gender	Male/Female
Karyotype	**T**risomy/**R**obertsonian translocation/**M**osaism
	Data per: **L**ab report/**M**edical chart/**P**arental Report
Ethnicity	**W**hite/**B**lack/**H**ispanic/**A**sian/**U**nknown

Table 2: Template of physical data. Recorded from all clinic visits for infants with DS in the first six months. Data from additional clinic visits were recorded in duplicate templates. Percentiles were found within chart as determine by growth charts and were not calculated.

Physical Data at Clinic Visit
Age at visit
Time 1 (days)
Occipitofrontal circumference (cm)
Occipitofrontal circumference (%tile)
Weight/Length (%tile)
Weight (g)
Height (cm)

Table 3: Template for cardiac data. Recorded from medical charts for infants with DS in the first six months. Bold letters indicate single letter codes used for simplicity in this study. CHD=chronic heart defect. Diuretics and Digoxin are both medications.

Cardiac
CHD (Y/N)
PFO/**A**SD/**V**SD/AV Cana**L**/**T**OF/Aortic **C**oarctation/P**D**A
Repair (Y/N)
If Yes, age (days)
Diuretics (Y/N)
Digoxin (Y/N)

Table 4: Template of gastrointestinal data. Recorded from medical records in the first six months for infants with DS. Bold letter indicate single letter codes used for simplicity. PPI=proton pump inhibitor, PEG=polyethylene (Miralax).

GI Tract
GI Abnormalities (Y/N)
Duodenal Atresia/Duodenal Stenosis/TEF/Malroation/Hirschprung
Repaired (Y/N)
Other: GER/Constipation/Delayed gastric emptying/Cow milk Protein intolerance
Medications: PPI/H2 blocker/Prokenetic/Senna/PEG

Table 5: Template of feeding data. Recorded from clinic visits or feeding evaluations (if done) in the first six months. Bold letters indicate single letter codes utilized. Times 1,2, and 3 correspond to clinic visits and subsequent data.

Feeding	
Route: **B**reastfed/Bo**T**tle/**N**G tube/**G**astric tube	
	Neonate: **B**/**T**/**N**/**G**
	Time 1
	Time 2
	Time 3
Type: **M**ilk/**F**ormula	
	Neonate: **M**/**F**
	Time 1
	Time 2
	Time 3
Required alterations: **T**hickened/Increased **C**aloric	
	Neonate: **T**/**C**
	Time 1
	Time 2
	Time 3
Feeding Evaluations (Results)	
	Age (corrected)
	Bedside/clinical:
	Swallow study (Y/N)
	Aspiration (Y/N)
	If yes: **T**hin/Thic**K**
	Penetration (Y/N)
	If yes: **T**hin/Thic**K**
	Desaturation (Y/N)
	Tachypnea (Y/N)
	Oromotor dysfunction (Y/N)
	Feeding refusal (Y/N)
	N-P regurgitation (Y/N)
	Other findings:

Table 6: Template for airway data. Recorded from polysonogram results in the first six months for infants with DS. Bold letters are single letter codes used for simplicity. Other than age, all results, if present, were found directly from PSG result report.

Polysonogram	
Study:	Age (corrected)
	Overnight PSG
	Nap PSG
	Apnea/Hypopnea Index (AHI)
	O2 min
	O2 mean
	EtCO2 max
	EtCO2 mean
	Respiratory rate range
	% REM sleep
	% AHI for REM sleep
	OSA (Y/N)
	If yes: **M**ild/Mo**D**erate/**S**evere
	Findings suggestive of dynamic UAO (Y/N)
	Number of Apnea events
	Mean second per apnea
	Apnea Index (AI)
	Number of hypopnic events
	Mean second per hypopnic event
	Hypopnic Index (HI)
	Total sleep time
	% Time spent in obstruction
	Other findings:

Table 7: Template of auditory data taken from medical records for infants with DS. Bold letters indicate single letter codes used for simplicity in study.

Auditory	
	Newborn Hearing Screen: **P**ass/**U**nilateral fail/**B**ilateral fail
	NBHS (if repeated): **P/U/B**
If refer: Follow up testing	
	Age (corrected)
Eval(s) performed	
	Otoacoustic Emission (OAE)
	Auditory Brainstem Response (ABR)
	Tympanogram:
	ENT evaluation
	Tympanostomy tubes placed (Y/N)

Table 8: Template of neurological and developmental data. Recorded from medical charts of infants with DS. Current gross motor skill was determined by medical professionals and recorded from clinic visits if calculated in the file.

Neurologic/Developmental:
Current gross motor skill level (months)
time 1 (months)
time 2 (months)
Corrected age (months)
time 1 (months)
time 2 (months)
Gross Motor Developmental Quotient

Table 9: General breakdown of birth information from all infants. Data from this study is compared to the Riley Neonate Unit and demonstrates similarities between DS birth information and that of non-DS neonates. n=119.

Trisomy 21	**Current Study**	**Riley Neonate**
Mean gestational age (wks)	37.7	33.9
Mean birthweight (g)	2996	2352
Mean body length (cm)	49.1	44.3
Mean Apgar score (1 min)	6.9	7
Mean Apgar score (5 min)	8.3	8
Percent White	66.4%	69.7%
Percent Hispanic	7.6%	8.7%
Percent Black	9.2%	15.3%
Percent Asian	3.4%	4%
Percent Unknown	13.4%	2.4%

Table 10: Other complications as found by videofluoroscopic evaluation. Data were recorded from evaluation reports. Demonstrate the high prevalence of additional feeding difficulties, n=64.

Other complications as found by video evaluation	n (%)
O_2 Desaturation	25 (39)
Tachypnea	3 (5)
Oral-motor dysfunction	16 (25)
Feeding refusal	6 (9)
Nasopharyngeal Regurgitation	14 (22)
Pharyngeal Pooling	34 (53)

Table 11: Comorbidities for infants with abnormalities in two organ systems. [1]Number of individuals with specific phenotype, [2]number and percentage of individuals with a trait in a single organ system, [3]percent of individuals with abnormality in the left column comorbid with trait above. CHD=chronic heart defect, n=119.

	Severe CHD (28)	GI (71)	Feeding (62)	Airway (43)	Auditory (32)	Endocrine (4)
Severe CHD (28)[1]	2 (7%)[2]	21 (75%)[3]	20 (71%)	13 (46%)	10 (36%)	1 (4%)
GI (71)	-	2 (3%)	47 (66%)	27 (38%)	23 (32%)	4 (6%)
Feeding (62)	-	-	2 (3%)	34 (56%)	14 (23%)	3 (5%)
Airway (43)	-	-	-	1 (2%)	12 (28%)	3 (7%)
Auditory (32)	-	-	-	-	1 (3%)	3 (9%)
Endocrine (4)	-	-	-	-	-	0 (0%)

Table 12: Correlations of cardiac or GI abnormalities with feeding strategy. For infants with feeding difficulty from feeding evaluation. Demonstrates the high correlation between the presence of either CHDs or GI abnormalities and difficulties feeding, both by evaluation or demonstrated by alterations to feeding route, n=50

	Early Oral to Late Oral	Early Oral to Late Tube	Early Tube to Late Tube	Early Tube to Late Oral
With feeding difficulty found in evaluation	15	10	16	9
CHD	13 (87%)	8 (80%)	13 (81%)	8 (89%)
ANY GI	9 (60%)	8 (80%)	12 (75%)	9 (100%)

Table 13: Listing of all structural GI defects recorded. Two have multiple abnormalities TEF=tracheoesophageal fistula. Other most often refers to the presence of an imperforate anus, n=18.

Structural Anomaly	n (%)
Duodenal Atresia	6 (5%)
Duodenal Stenosis	1 (0.8%)
TEF	0 (0%)
Malrotation	1 (0.8%)
Hirschsprungs	7 (5.9%)
Other	5 (0.4%)

Table 14: Details regarding apneas and hypopneas from PSGs. Both number of occurrences and indices were recorded directly from PSG reports, not calculated. PSG=polysonogram, n=44.

Apneas and Hypopneas as found by PSG	
Mean Number Apneas	44.83
Mean Apnea Index (AI)	17.18
Mean Number Hypopneas	15.81
Mean Hypopnea Index (HI)	11.35

FIGURES

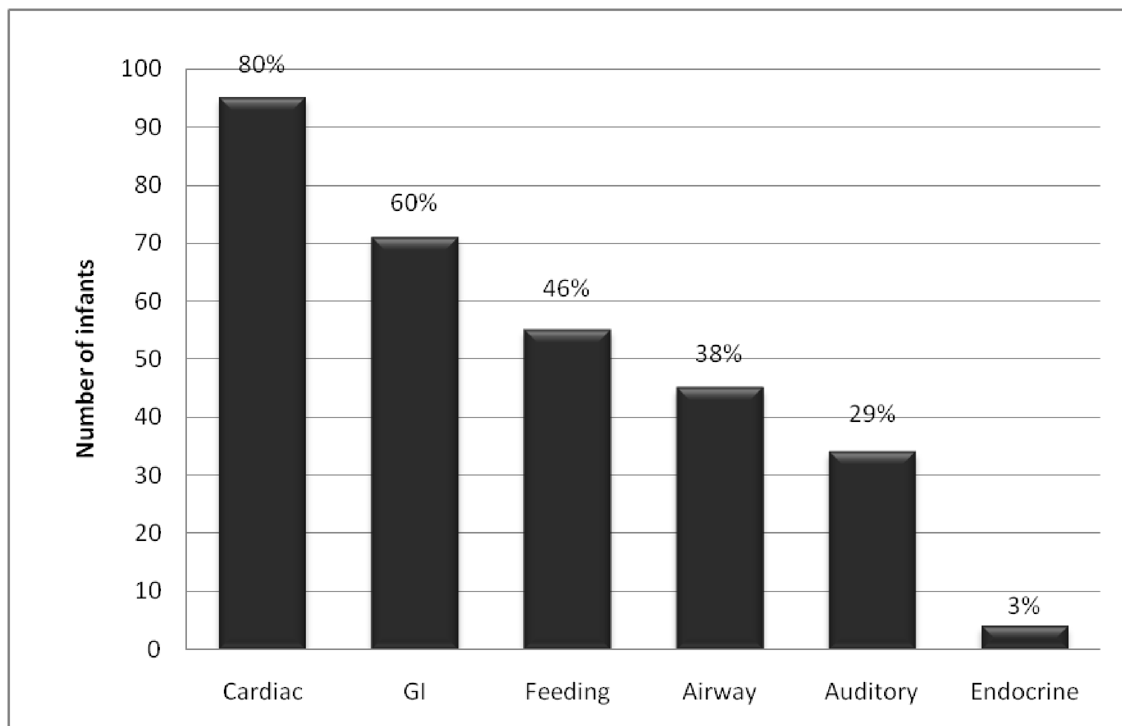


Figure 1: Comprehensive results from a single organ system. Includes data from all infants and percentages are taken as such.

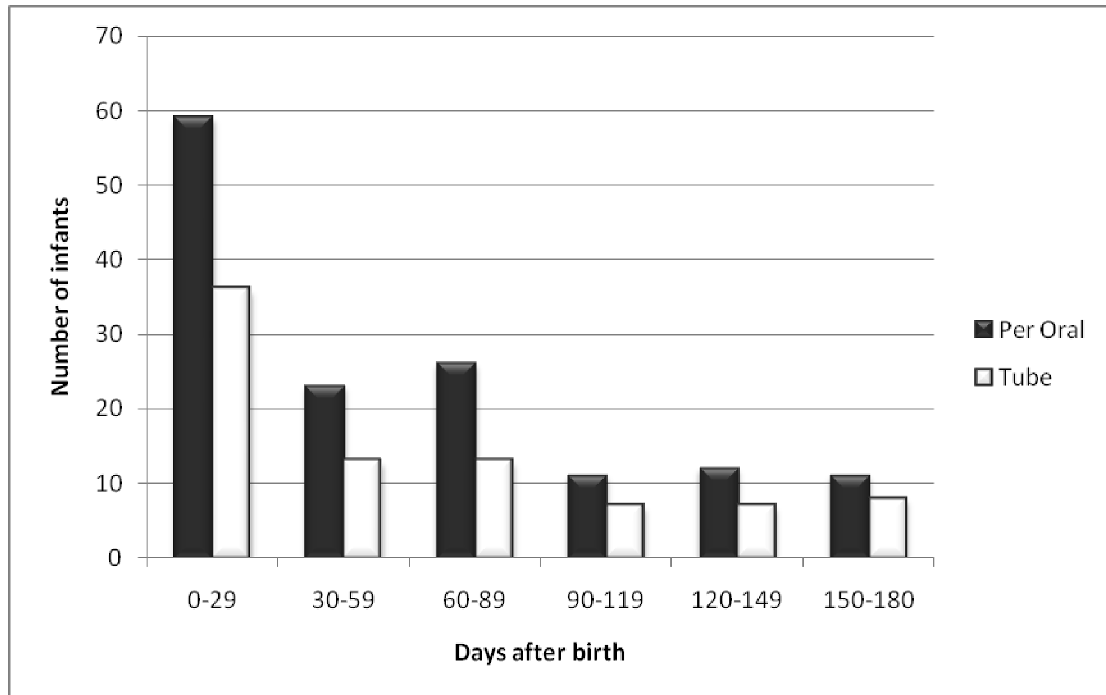


Figure 2: Feeding route for all infants with at least one clinic visit over time. Per oral is defined as feeding by breast or bottle and tube as feeding by nasogastric tube or gastronomy tube, n=115

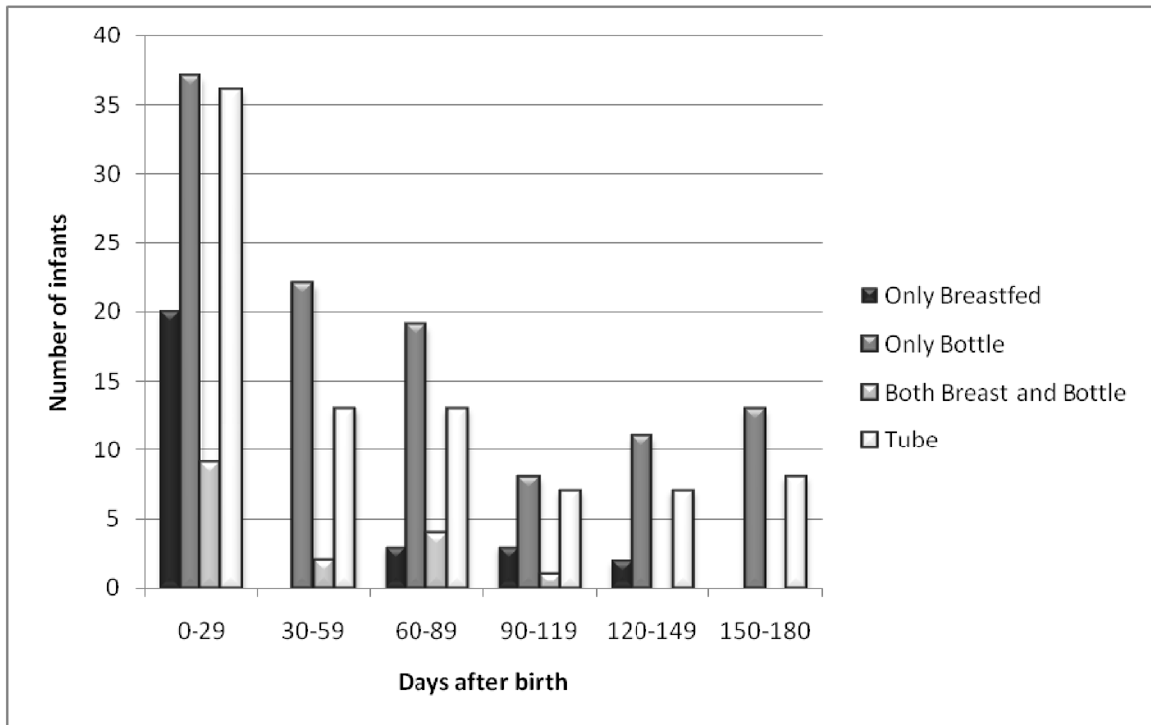


Figure 3: Breakdown of feeding route for all infants with at least one clinic visit. Depicts how infants were feeding when they visited the clinic in days after their birth, n=115

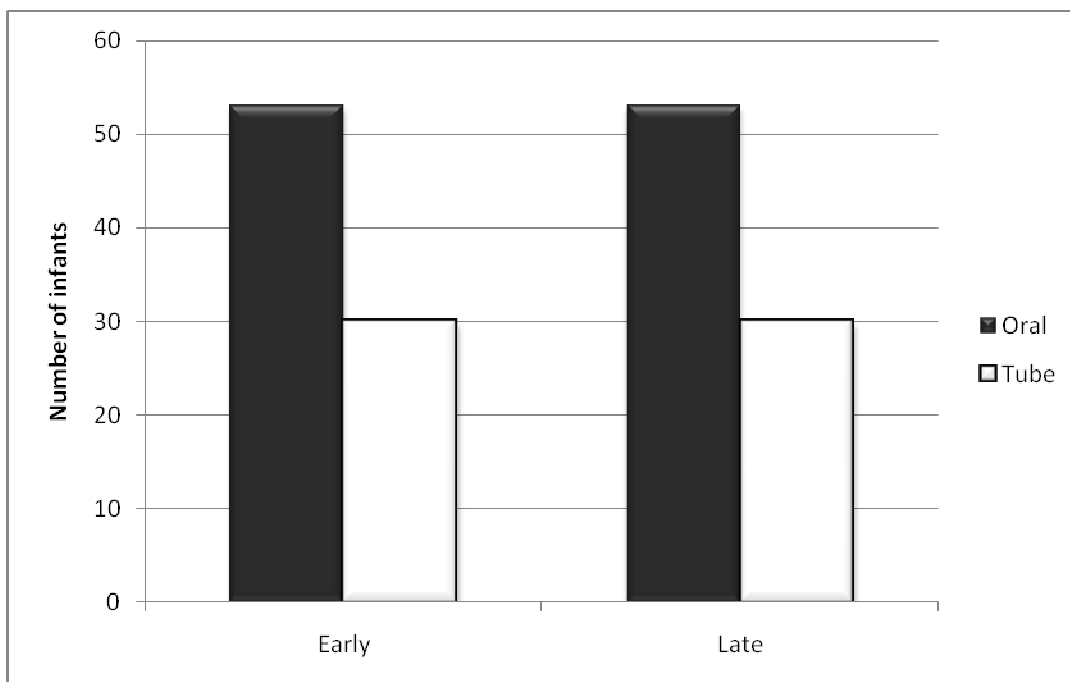


Figure 4: Feeding route for infants with at least two visits, early and late, a comparison of the earliest and latest clinic visits. Oral is defined by breast or bottle feeds and tube as nasogastric or gastronomy tubes. Early is defined as first visit to the clinic and late as last visit to the clinic documented in the first 6 months of life, n=83.

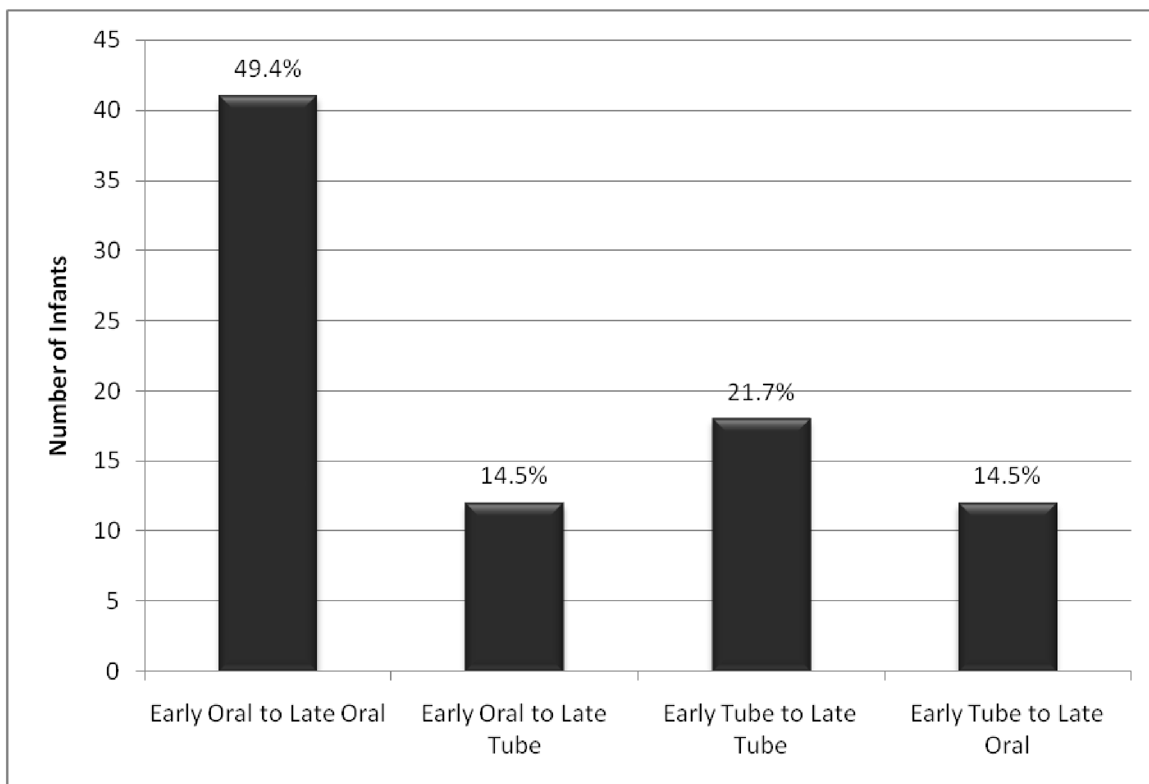


Figure 5: Alteration of feeding strategy in infants with at least two visits. Percentages are of infants with two visits. Demonstrates the changes in feeding strategy in the first 6 months of life where early is defined as the first visit to the clinic and late as the last visit documented in the first 6 months, n=83.

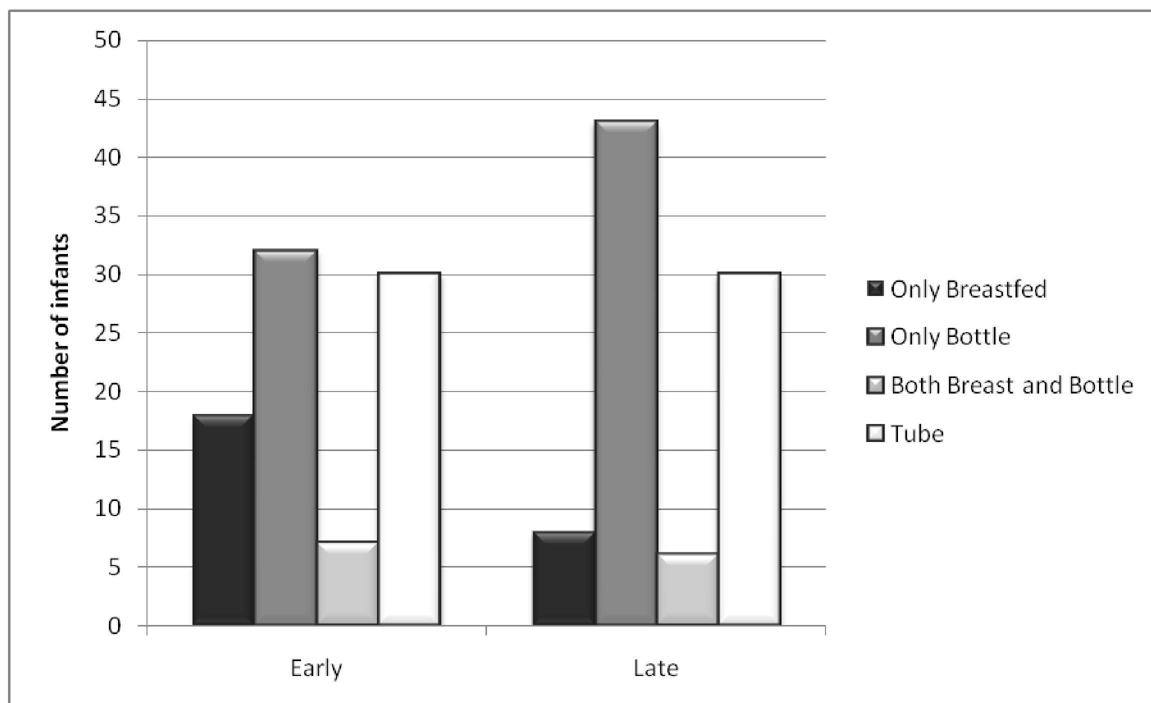


Figure 6: Specific feeding route for infants with at least two visits, early and late. Demonstrates a change how infants were feeding between early, first visit, and late, last visit in the first 6 months, n=83.

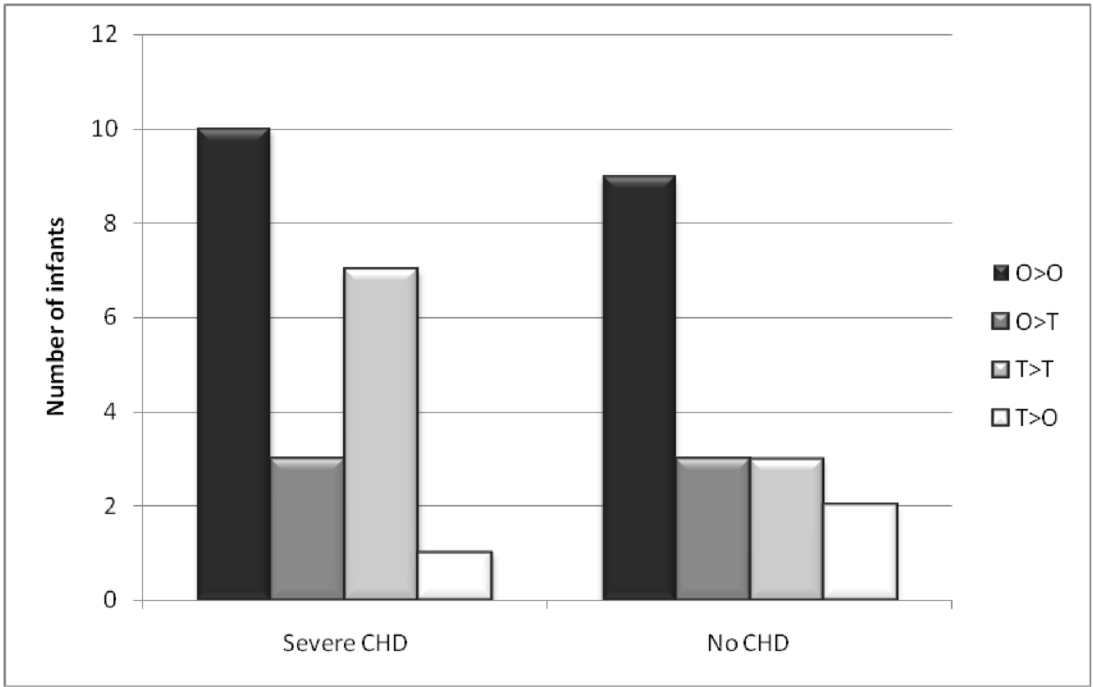


Figure 7: Altered feeding strategy correlated with cardiac defects. CHD=congenital heart defect. Severe CHD=defects that required repair or medication. O>O=early oral to late oral, O>T=early oral to late tube, T>T=early tube to late tube, and T>O=early tube to late oral, (Severe, n=28; No CHD, n=24).

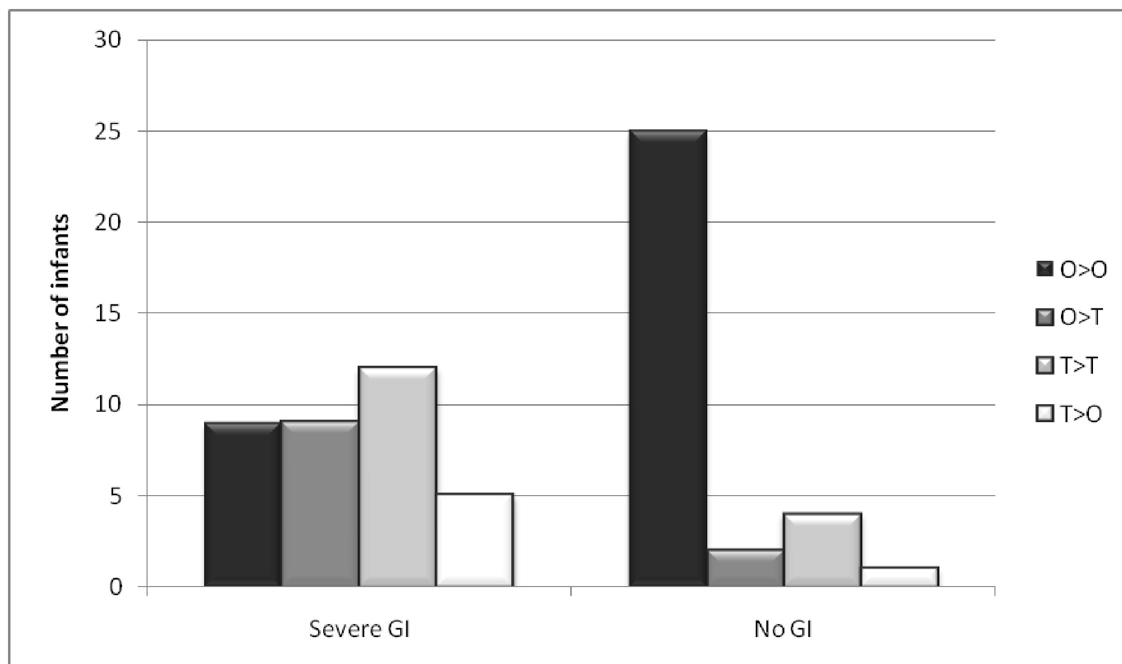


Figure 8: Altered feeding strategy correlated with GI abnormalities. GI=gastrointestinal. O>O=early oral to late oral, O>T=early oral to late tube, T>T=early tube to late tube, and T>O=early tube to late oral, (Severe, n=48; No GI, n=48).

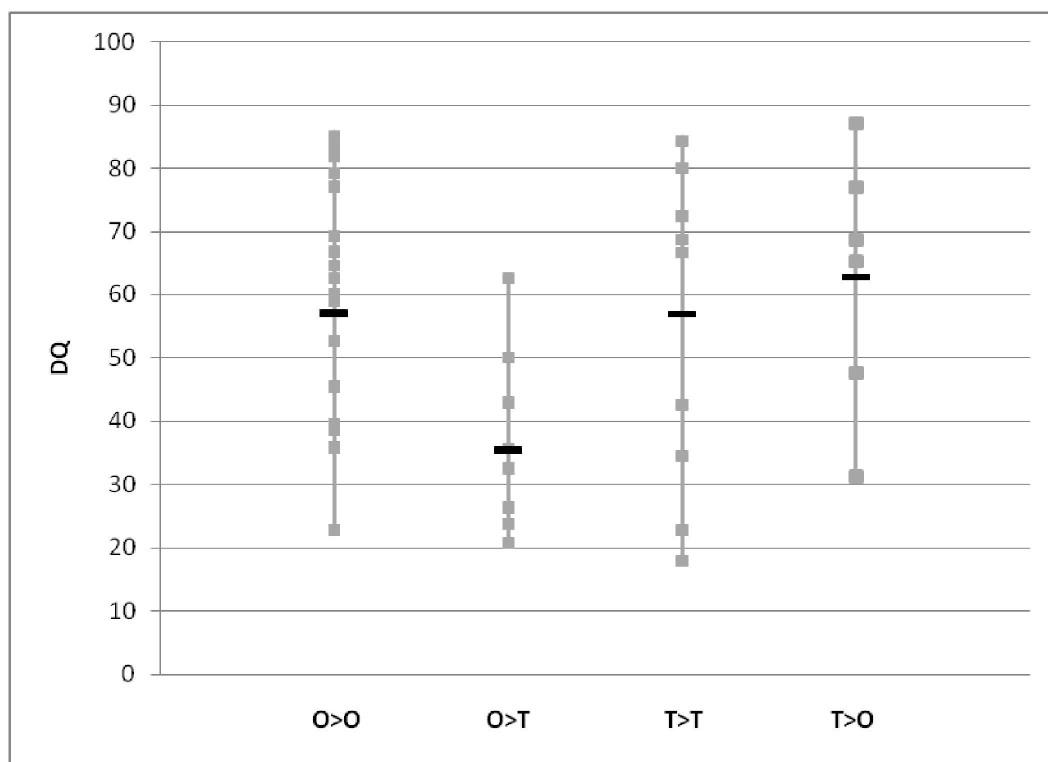


Figure 9: DQs for infants with altered feeding strategies and at least two visits. Black bars indicate averages. O>O=early oral to late oral, O>T=early oral to late tube, T>T=early tube to late tube, and T>O=early tube to late oral, n=44.

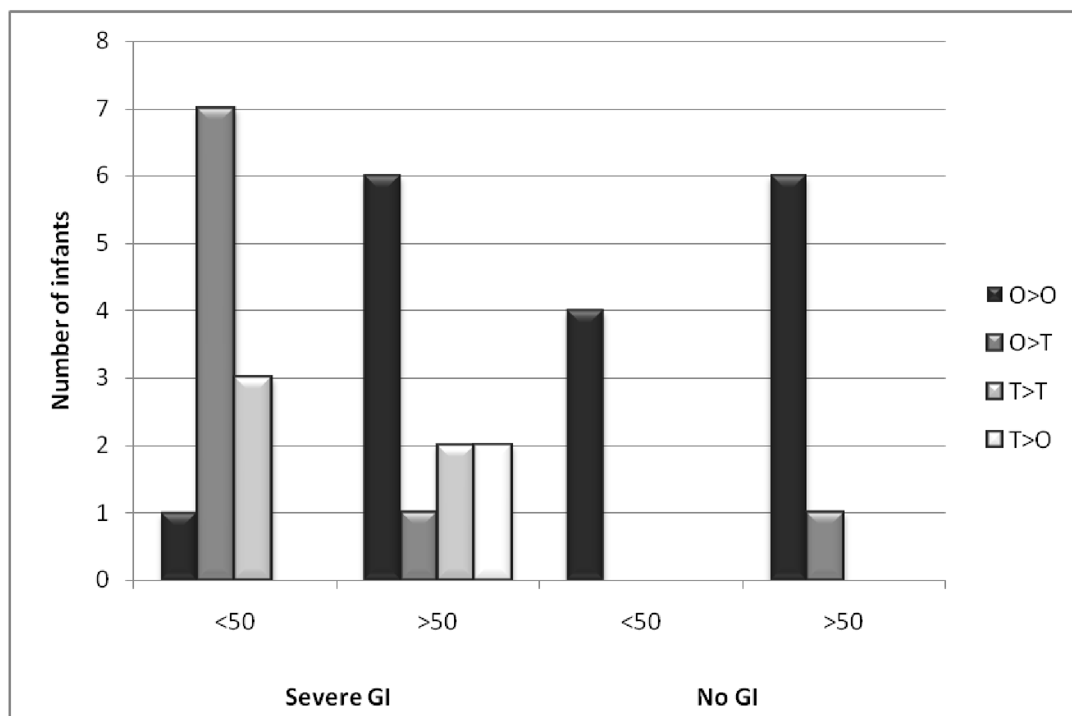


Figure 10: DQs for infants with altered feeding strategies in the presence or absence of a severe GI abnormality. O>O=early oral to late oral, O>T=early oral to late tube, T>T=early tube to late tube, and T>O=early tube to late oral, n=33.

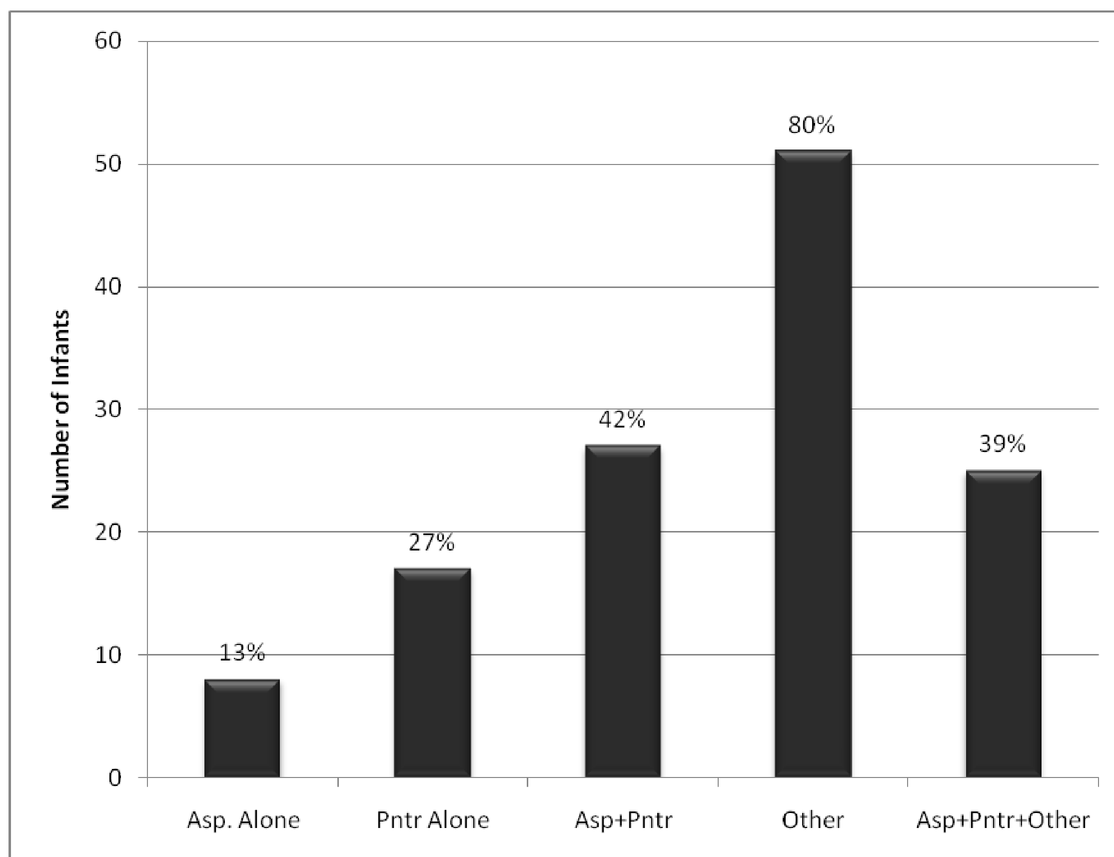


Figure 11: Results from feeding evaluations for infants with DS, and percentages of total number of infants. Asp=aspiration. Pntr=penetration. Other defined as complications found during feeding evaluations such as oxygen desaturation, pharyngeal pooling, and oral-motor dysfunction, n=64.

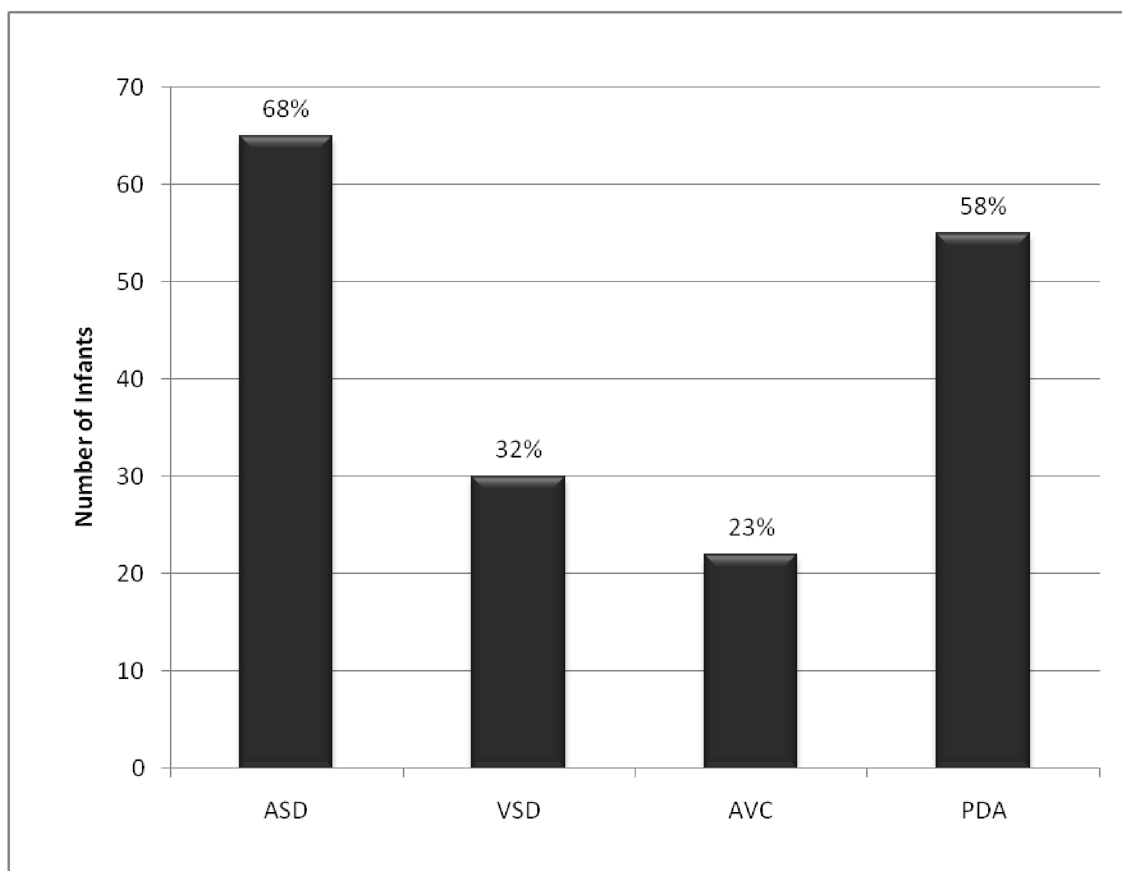


Figure 12: Occurrences of cardiac defects. The number of infants found to have the defect recorded in the first six months with the percentage of those with cardiac defects. ASD=atrial septal defect, VSD=ventral septal defect, AVC=atrioventricular canal defect, PDA=patent ductus arteriosus. Percentages taken of those with heart abnormalities, n=95.

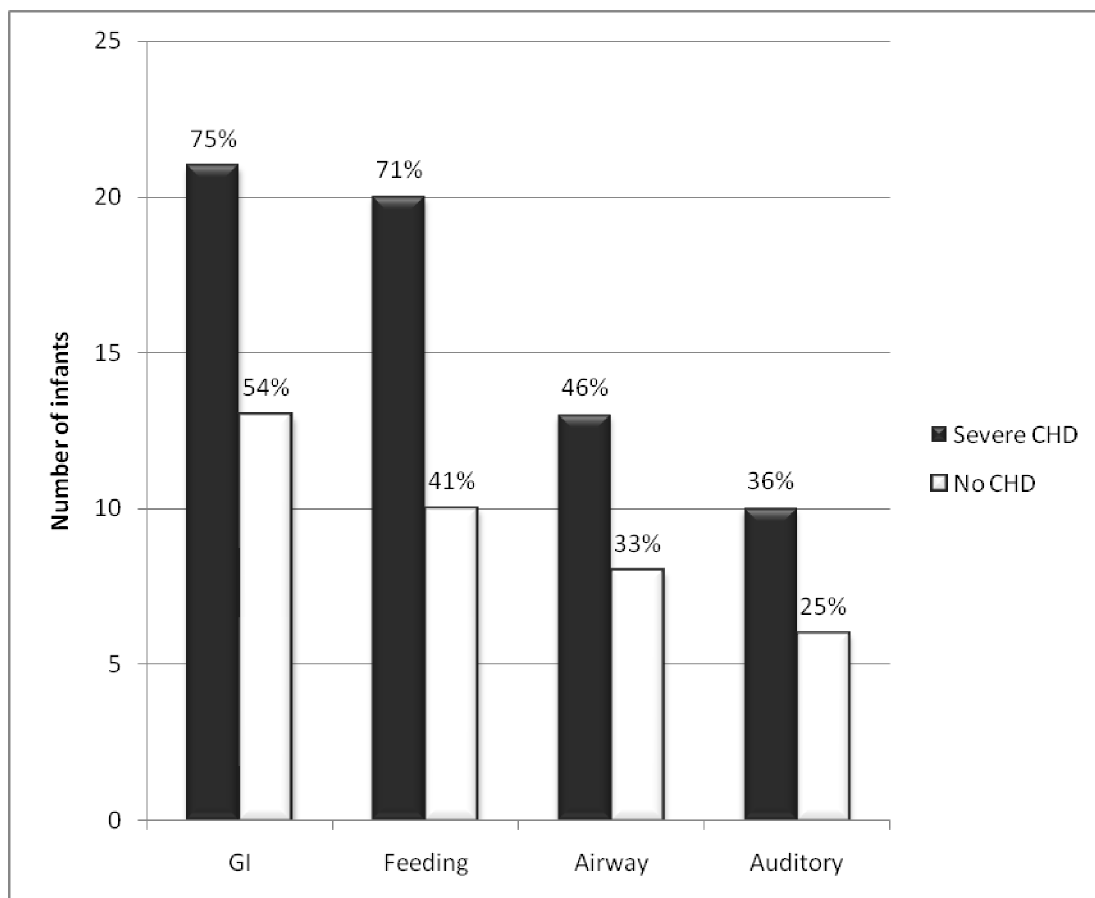


Figure 13: Comorbidities of two systems to compare the presence of a severe cardiac defect with the absence of a defect. Percentages are taken of the specific population (eg. 75% of those with a severe CHD have a GI anomaly). CHD=congenital heart defect. GI=gastrointestinal. Severe CHD=heart defect that required surgical repair or medication. Severe CHD, n=28; No CHD, n=28.

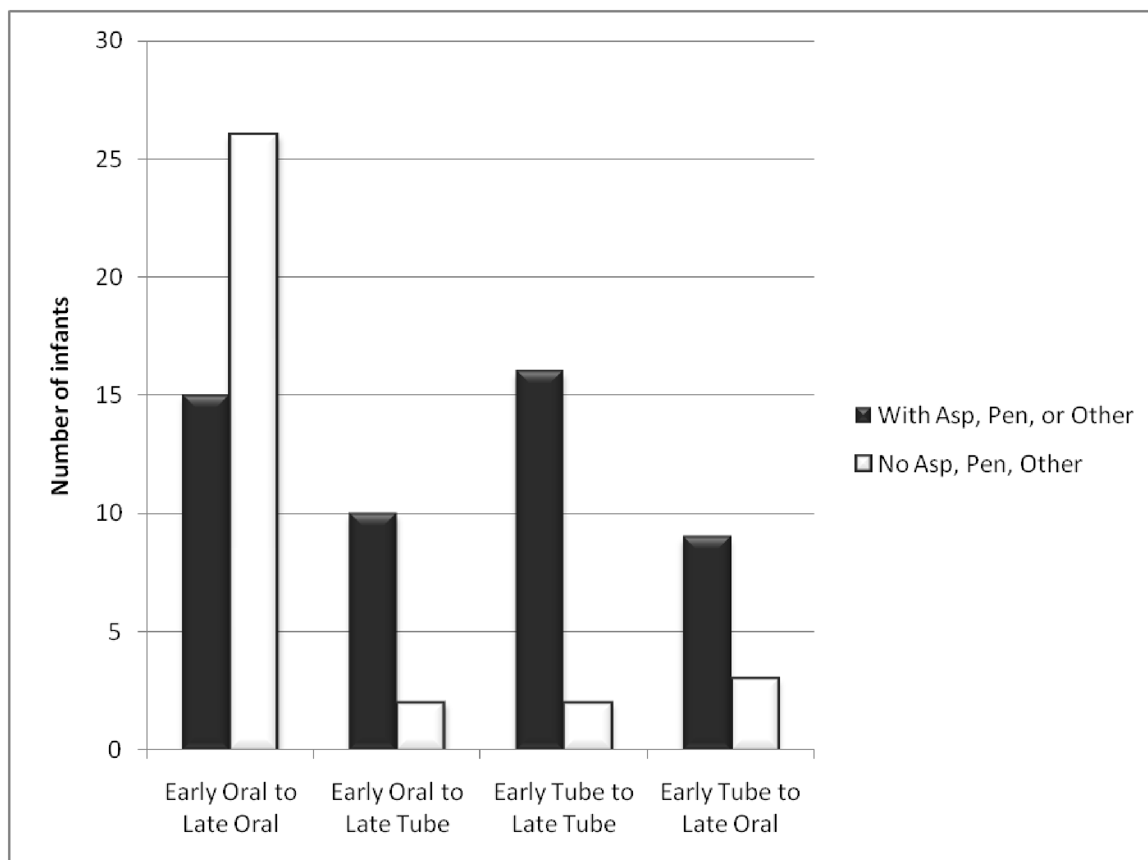


Figure 14: Alteration of feeding strategies of those with or without feeding difficulties. Asp=aspiration, Pntr=penetration, Other is defined by additional complications found by feeding evaluation such as oxygen desaturation or oral-motor dysfunction. Early is defined as first visit to the clinic and late as last visit to the clinic documented in the first 6 months of life, n=83.

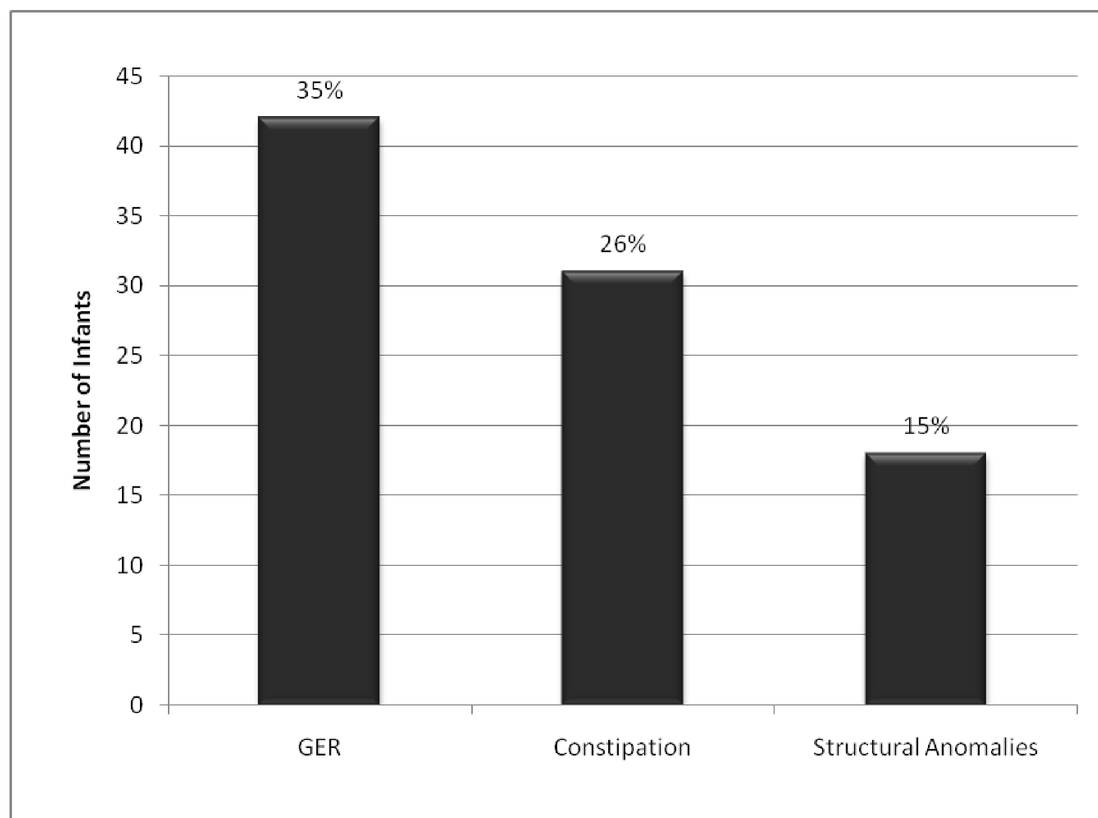


Figure 15: Occurences of functional and structural GI abnormalities. The number of infants recorded to have any GI abnormality and the percentage taken of all infants. GER=gastroesophageal reflux, n=119.

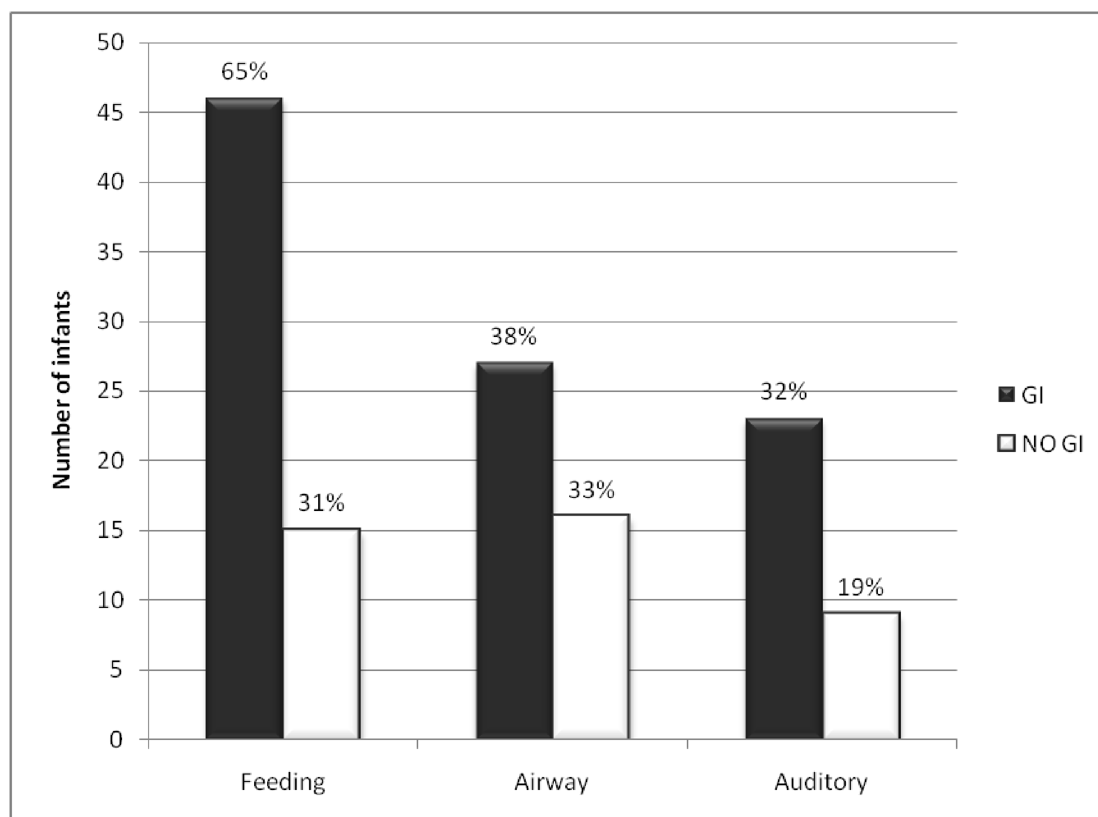
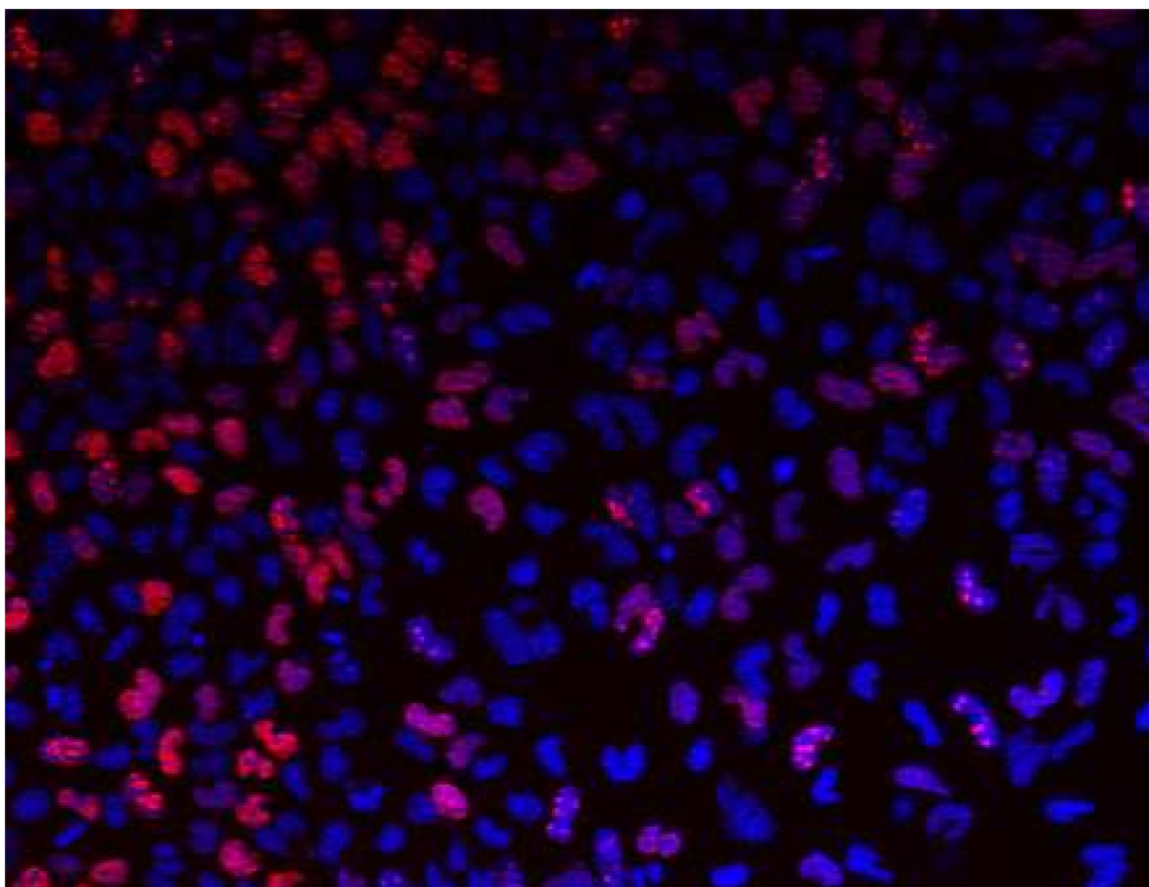


Figure 16: Comorbidities of two systems to compare the presence of a GI abnormality with the absence of an abnormality. Percentages are taken of the specific population (eg. 65% of those with a GI anomaly have a feeding difficulty). GI=gastrointestinal. GI, n=71; No GI, n=48.

A.



B.

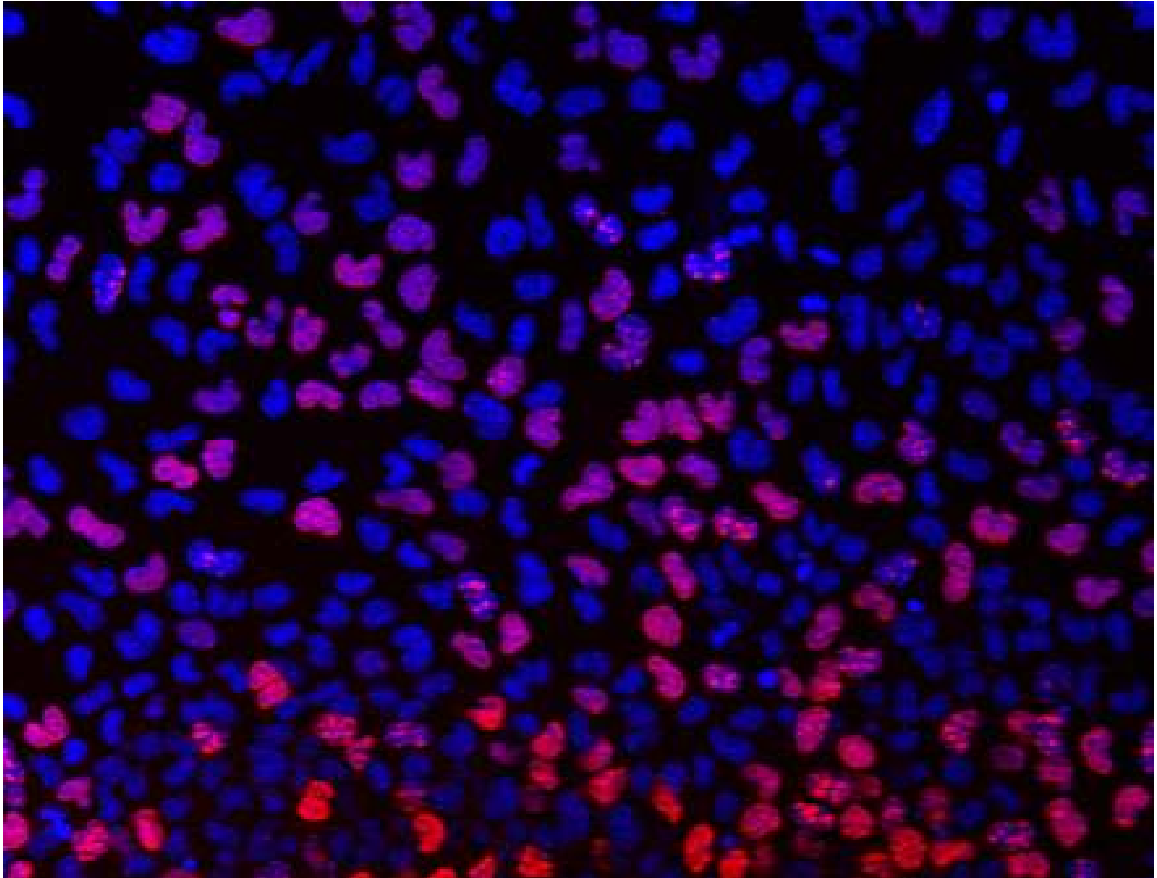


Figure 17: Proliferation assay of E10.5 NC from BA1s one day after dissection. Blue is DAPI, red is a modified Edu assay (Click-iT EdU) illustrating those cells undergoing proliferation. Both demonstrate approximately 45% of cells still proliferating. A. Euploid, B. Trisomic

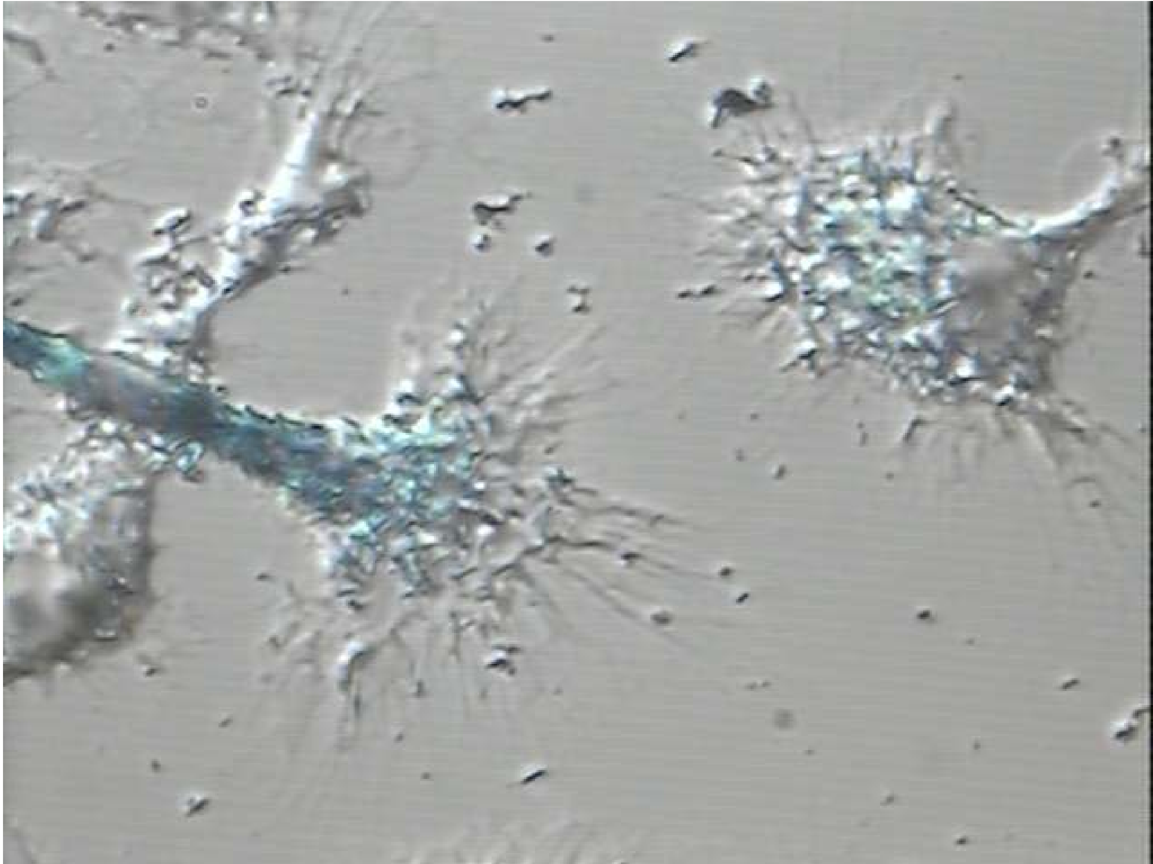


Figure 18: SA-βgal activity of senescent cells (BJ line). Blue illustrates SA-βgal activity and senescent cells. Picture at 100X

PUBLICATION

PCR prescreen for genotyping the Ts65Dn mouse model of Down syndrome

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Down syndrome (Trisomy 21, or DS) is the most common live-born aneuploidy in humans occurring in approximately 1 of 700 live births (1). Inheritance of three copies of human chromosome 21 (Hsa21) results in a wide range of phenotypes including cognitive impairment, cardiac defects, and craniofacial dysmorphology (2,3). Conserved gene content and order occurs between Hsa21 and murine chromosomes 16 (Mmu16), Mmu17, and Mmu10 (4-6). To better understand the gene-phenotype relationship in DS, partial or segmental trisomies were created in mice using small translocation chromosomes which triplicated portions of the murine genome that correspond to Hsa21 (7). Numerous phenotypes associated with DS have been documented in mouse models (recently reviewed in (8-10)).

The most widely used and well studied mouse model of trisomy and DS phenotypes is the Ts(17^{16})65Dn (hereafter Ts65Dn). To create this mouse, DBA/2J male mice with irradiated testes were bred to C57BL/6J female mice, and offspring carrying reciprocal translocations for Mmu16 were bred to B6C3F1 mice (11). The resultant T(16C3-4;17A2)65Dn heterozygote mice produced offspring with the small marker chromosome consisting of the telomeric 13.5 Mb of Mmu16, attached to a small Mmu17 centromere with a small amount of euchromatic material (5-7,12). Ts65Dn mice are trisomic for orthologs of approximately half of the genes on Hsa21 and show DS-related phenotypes including reduced birth weight, perinatal lethality, craniofacial abnormalities, cognitive and behavioral impairments, cardiovascular malformations, and neurological structural deficiencies (10,13-17). Investigations using this mouse model have been used to predict human phenotypes as well as to examine potential therapies to correct traits associated with DS (9,10,13).

Ts65Dn mice serve as an excellent genetic and phenotypic model for DS, however, they are difficult to produce and genotype. Generation of Ts65Dn mice is complicated given that males are subfertile, females are poor mothers, and offspring are limited due to perinatal loss (7,16,18). Physical differences have been observed between offspring containing the segmental trisomic and euploid littermates, but these phenotypes are highly variable and not reliable for positive identification of Ts65Dn mice (12,16). Ts65Dn mice were first genotyped using karyotypes from cultured lymphocytes or meiotic cells, a procedure taking 2-3 days and only done in adult mice (11). Fluorescence *in situ* hybridization (FISH) of nucleated blood cells at interphase has been used as the standard for identifying Ts65Dn mice but this method is also labor intensive, and generally requires 2-3 days (19). Recently a number of quantitative PCR methods have also been used to quantify trisomic gene copy number in Ts65Dn mice (20-23). While this process is relatively fast, qPCR can be temperamental, expensive, and few laboratories have the necessary equipment, requiring the service of a core facility (22). Erroneous inclusion of euploid mice as trisomic in assessment of a subtle phenotype can significantly reduce the precision of subsequent phenotyping procedures.

To prescreen Ts65Dn mice and reduce the time and cost of subsequent FISH identification of the trisomic segment, we have developed a simple methodology requiring the restriction digest of a PCR product amplified from the small translocation marker chromosome (17^{16}). This process is inexpensive, identifies trisomic mice with more than 90% concordance to FISH genotyping, can be done in most laboratories, and takes approximately six hours to complete (instrument run times and incubations take the majority of this time). Our methodology utilizes a single nucleotide polymorphism

(SNP) within the *Zdhhc14* gene on Mmu17 near the breakpoint of the translocation chromosome. The SNP is polymorphic between the DBA reciprocal translocation founder strain and the C57BL/6J and C3H/HeJ strains that contribute to the majority of the background of Ts65Dn mice. Crosses between Ts65Dn and B6C3F1 mice result in offspring with two normal Mmu17 with C57BL/6J or C3H/HeJ alleles. In most cases, trisomic offspring will have the DBA *Zdhhc14* allele at the Mmu17 centromeric end of the chimeric chromosome due to limited crossovers during meiosis within the region delimited by the SNP and the $Mmu17^{16}$ breakpoint. PCR amplification of the area surrounding the SNP allows for the selective digest of C57BL/6J or C3H/HeJ product by *Sac*I, which produces bands of 175 and 71 bp in length. Ts65Dn mice with the DBA allele at the SNP will display a full length PCR product (246 bp) in addition to the digested products from the additional copies of Mmu17 with C57BL/6J or C3H/HeJ alleles (The 71 bp band can be difficult to visualize on an agarose gel) (Figure 2).

In total, 1174 offspring from Ts65Dn x B6C3F1 matings were genotyped using this PCR and restriction enzyme methodology. Of those, 716 were sexed and were comprised of 50.3% males and 49.7% females. At weaning, 32.7% of 892 offspring carried the distinguishing DBA *Zdhhc14* allele on the translocation chromosome. Both positive and negative PCR results from 603 pups were confirmed using FISH with an overall 3.1% false positive rate and a 5.2% false negative rate (Table 1). Of the 338 female mice that were genotyped using PCR, 152 were confirmed by FISH results. This includes 51 mice that were PCR negative for trisomy, but were verified using FISH either because of their small size or use as a control. Using the prescreen on PCR positive females alone can reduce the time and cost of FISH analysis by approximately 66%, and

50% if some PCR negative mice are elected to be analyzed by FISH to ensure acquiring the 5% of false negatives. The methodology can only prescreen Ts65Dn offspring due to small frequency of recombination events between the small centromeric end of Mmu17 on the marker chromosomes (originally derived from DBA/2J) and corresponding regions on the two full length copies of Mmu17 (largely derived from the B6C3F1 males after generations of crossing onto this background). We have utilized this methodology in our colonies as a cost effective and efficient method to prescreen Ts65Dn mice for experimental procedures and continued mating, with subsequent FISH identification of the small marker chromosome.

ACKNOWLEDGEMENTS

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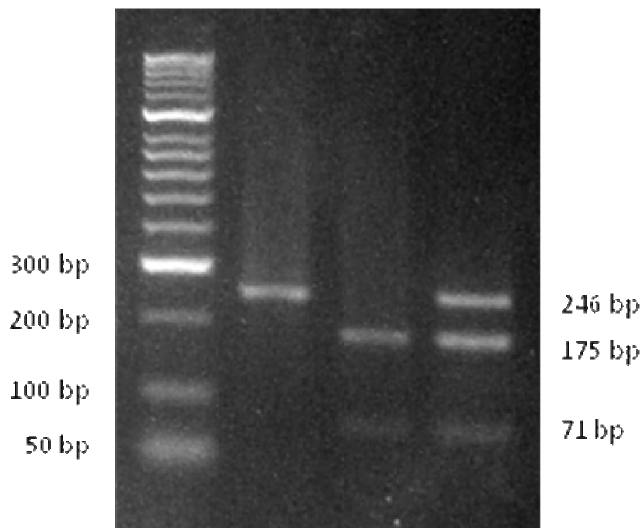
Table 1: **FISH confirmation of PCR genotyped Ts65Dn offspring**

Age	Status	N	FISH Positive	FISH Negative	False Type
P0	PCR positive	59	59	0	0%
P0	PCR negative	93	4	89	4.3%
P5-P10	PCR positive	168	159	7	4.2%
P5-P10	PCR negative	119	7	112	5.9%
P21-P30	PCR positive	70	68	2	2.9%
P21-P30	PCR negative	153	8	145	5.2%

Figure 1: **Site of *Zdhhc14* polymorphism relative to primers.** Location of SNP and *Sac*I restriction endonuclease site (BLUE) between primer sequences (RED) utilized for the PCR and restriction digest protocol.

5′ – **AAATAGTAGCATCTCATGAGTG**TTGACGGAGCTGGACCCTTGGCC
CTGCAGATTCCTTGGGCCCCGA**GAGCKC**CCTTGTTTCGGGTCATCCTGGAT
ACAAAGAGAGAGGCTGTCTGTACCCTTAGACTTTAAAGAAGACACTGGATG
CTTATCAGCAAGTGTTCTGATGCATCCTGTTGATAAAAGTATCTGGTGGGTTG
GGGGTGTAGCTCAGTGTGCAATGG**GCTTGTCTAAGATGCACTATG** – 3′

Figure 2: **PCR/restriction digest typing for the T65Dn marker chromosome.** Lane 1) Molecular weight markers; Lane 2) a 246 bp PCR fragment is produced on all three backgrounds (B6, C3H, DBA); Lane 3) in euploid mice, both alleles contain the *Sac*I restriction digest products of 175 bp and 71 bp; and Lane 4) Ts65Dn offspring have one copy of the 246 bp band from the T65Dn marker chromosome, plus the 175 and 71 bp bands from the parents.



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SUPPLEMENTAL MATERIAL

Materials and methods

Mice

Female B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn) and male B6 x C3H/HeJ F_1 (B6C3F1) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Offspring from Ts65Dn x B6C3HF1 matings were produced in our colonies at Indiana University-Purdue University Indianapolis (IUPUI) and the Johns Hopkins University School of Medicine (JHUSOM). All male/female pairs are kept in a 12 hr day/12 hr night light cycle with food and water administered *ad libitum* in a temperature and humidity controlled room. Animal use and protocols were approved by the IACUC committees at IUPUI and JHUSOM.

DNA Isolation

Tissue was obtained from offspring of Ts65Dn mothers at postnatal day zero (P0), 5-10 (P5-10) or 21-30 (P21-30), and pups were marked for subsequent identification. DNA was isolated using standard protocols (overnight Proteinase K digestion and isolation the following day by salting out). In our hands, the commonly used "HotSHOT" (25) method did not reliably produce DNA suitable for the subsequent PCR.

PCR

PCR was utilized to amplify a 246 bp segment with a single nucleotide polymorphism (SNP) in the *Zdhhc14* gene (rs48029645 GenBank) using the primers (forward) 5'-AAATAGTAGCATCTCATGAGTG-3' and (reverse) 5'-CATAGTGCATCTTAGACAAGC-3'(T_m=60°C) (Invitrogen, Carlsbad, CA) (Figure 1).

For a single reaction, 1X PCR Rxn Buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), and 0.02 μg/μL TaqDNA Polymerase (Bioline, Taunton, MA) were used with 2 μL of DNA (approximately 100 ng/μL) in a 25 μL reaction. DNA was amplified using the following steps: 1) 94°C for 1 min, 2) 94°C for 30 sec, 3) 65°C for 20 sec, 4) 72°C for 20 sec, 5) repeat steps 2-4 5 cycles, 6) 94°C for 30 sec, 7) 65°C for 30 sec (-1 degree per cycle), 8) 72°C for 1 min, 9) repeat steps 6-8 10 cycles, 10) 94°C for 30 sec, 11) 55°C for 30 sec, 12) 72°C for 20 sec 13) repeat steps 10-12 10 cycles,14) 72°C for 1 min, 15) 72°C hold. Afterwards, 8 μL of the PCR can be removed and a 246 bp band visualized on a 1% agarose gel (Figure 2).

Restriction Digest

The *Sac*I enzyme was used to discern the SNP in PCR products. For a single reaction, 2 μL of Buffer I (New England Biolabs, Ipswich, MA), 2 μL of 10X BSA, 7 μL sterile water, and 1 μL of *Sac*I restriction enzyme were combined in a 1.5 mL eppendorf tube to which 16 μL of the PCR product was added. Samples were incubated in a 37°C water bath for 2.5 hours and resolved on a 3% agarose gel, Products were visualized using SYBR Safe DNA gel stain (Invitrogen) or ethidium bromide and a UV source.