Craniosynostosis, Fibroblast Growth Factor Receptors and Gastrointestinal Malformations – A Possible Link

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Dentistry
University of Toronto

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2014

Abstract

Syndromic craniosynostosis is most commonly associated with mutations in Fibroblast Growth Factor Receptor genes (FGFR)-1, 2 and 3. Clinical and animal reports suggest a link between FGFR-associated craniosynostosis and defects in the gastrointestinal tract (GIT).

**Objective:** to determine whether GIT malformations occur more frequently in the craniosynostosis population with a known FGFR mutation when compared to the general population.

**Methods:** A retrospective chart review of patients diagnosed with Crouzon, Pfeiffer or Apert syndromes between 1990 and 2011 was performed at the Hospital for Sick Children in Toronto. Thirty-two charts meeting inclusion criteria were analyzed for any history of GIT abnormalities.

**Results:** Three out of 32 patients had documented intestinal/bowel malrotations while 7 had gastroesophageal reflux disease. All patients had documented FGFR2 mutations, a finding in line with previous studies and published case reports.

**Conclusions:** Results suggest an association between FGFR-associated craniosynostosis and GIT malformations.
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<td>Apert syndrome</td>
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<tr>
<td>Arginine</td>
<td>Arg</td>
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<tr>
<td>Base pairs</td>
<td>bp</td>
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<td>Central nervous system</td>
<td>CNS</td>
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<td>Cleft palate</td>
<td>CP</td>
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<tr>
<td>Crouzon syndrome</td>
<td>CS</td>
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<tr>
<td>Crouzon syndrome with acanthosis nigricans</td>
<td>CS-AN</td>
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<td>Cysteine</td>
<td>Cys</td>
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<td>Extracellular signal-regulated kinases</td>
<td>ERKs</td>
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<td>European Surveillance of Congenital Anomalies</td>
<td>EUROCAT</td>
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<tr>
<td>Fibroblast Growth Factor</td>
<td>FGF</td>
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<td>Fibroblast Growth Factor Receptor</td>
<td>FGFR</td>
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<td>Gastroesophageal reflux disease</td>
<td>GERD</td>
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<tr>
<td>Gastrointestinal</td>
<td>GI</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>GIT</td>
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<tr>
<td>Heparan sulfate proteoglycan</td>
<td>HSPG</td>
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<tr>
<td>Hospital for Sick Children</td>
<td>SickKids</td>
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<tr>
<td>Immunoglobulin</td>
<td>Ig</td>
</tr>
<tr>
<td>Left-right</td>
<td>L/R</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>MAP</td>
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<tr>
<td>Pfeiffer syndrome</td>
<td>PS</td>
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<tr>
<td>Phenylalanine</td>
<td>Phe</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
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<td>-----------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>PKC</td>
</tr>
<tr>
<td>Transforming growth factor</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyrosine kinase</td>
<td>TK</td>
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<td>Serine</td>
<td>Ser</td>
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I. INTRODUCTION AND LITERATURE REVIEW

Craniosynostosis is a condition characterized by the premature fusion of one or more cranial sutures. Craniosynostosis may be isolated or as part of multisystem syndromes. When a child is suspected to have a craniosynostosis syndrome they may require further diagnostic and genetic tests as these individuals often have a higher risk of other physical and developmental conditions. The genetic cause of syndromic craniosynostosis is heterogeneous; however, the most commonly mutated genes are the *Fibroblast Growth Factor Receptor (FGFR)* genes, specifically *FGFR1*, *FGFR2* and *FGFR3*.

Several published reports present gastrointestinal (GI) malformations, in particular intestinal malrotations, as comorbidities associated with syndromic craniosynostosis. These reports, along with evidence from mouse models, suggest an association. Expression of the *FGFRs* in the GI tract (GIT) further strengthens a possible genetic link between FGFR-associated craniosynostosis syndromes and the presence of GI defects in these patients. This study is focused on analyzing the incidence of specific GI defects in craniosynostosis patients with known mutations in the *FGFR* genes. This section will begin with a brief review of the formation of cranial sutures, followed by the incidence, types, causes, and management of craniosynostosis and a discussion of the associated syndromes. The genetic basis of FGFR-associated craniosynostosis and the related GIT changes will be discussed in the following sections.

1. CRANIOSYNOSTOSIS

1.1 Cranial sutures – development and fusion:

The neurocranium is composed of flat calvarial bones and a cranial base (Fig.1). Intramembranous bone formation is responsible for generating the flat calvarial bones that give rise to the roof and sides of the skull whereas the cranial base develops by endochondral bone formation. Needle-like bone spicules radiate peripherally at the margins of the frontal, parietal and occipital bones to produce the fontanelles and sutures (Cohen, 2002). Fontanelles are soft membranous gaps found between incompletely formed cranial bones on an infant’s skull.
Sutures develop at the periphery of the extending bone fields, due to a wedge-shaped proliferation of cells, referred to as osteogenic fronts (Decker & Hall, 1985). It is these osteogenic fronts that seem to direct the morphogenetic determination of sutural patterning. The osteogenic fronts can approach each other in one of two ways, yielding either an edge-to-edge suture or a beveled suture (Johansen & Hall, 1982). End-to-end sutures form in the midline of the skull with both the sagittal and metopic sutures being formed this way (Kokich, 1976). Coronal, lambdoidal and frontozygomatic sutures are formed away from the midline and form a beveled suture (Cohen, 2002). It has been proposed that the end-to-end suture is the result of biomechanical forces equal in magnitude acting on each opposing side of the midline and initiating suture formation while maintaining the flat calvarial bones in the same plane of space (Cohen, 2002). In contrast, sutures formed away from the midline are likely to be affected by unequal biomechanical forces, thus moving the bones into different planes of space yielding an overlapping, beveled suture (Cohen, 2002). Fusion of the sutures located along the cranial midline, such as the sagittal and metopic sutures, seem to produce more prominent ridging compared to the ridging observed with synostosis of the sutures occurring away from the midline such as the coronal and lambdoidal sutures (Cohen, 2002).

The timing of fusion of these sutures follows a strict temporal pattern, with most of the sutures normally fusing after birth. In infancy, flexible sutures and open fontanelles allow for deformation of the calvarium by enabling the bones of the skull to overlap as an infant passes through the birth canal during delivery without damaging the infant’s brain (Levi et al., 2012). During childhood, the brain grows and expands rapidly. Patent sutures allow the brain to grow quickly without being constricted, while still providing a protective shell for the brain. The fontanelles are the first to close during infancy and early childhood, followed by sutural fusion in early adulthood when growth is complete (Cohen, 2002). The closure of the posterior fontanelle generally occurs by the third month of life, while the anterior fontanelle typically closes between the ages of 7-19 months (Aisenson, 1950). The metopic suture is the only suture that fuses early on in life, undergoing fusion between six to eight months of age (Weinzweig et al., 2003). All other cranial sutures usually begin to close when an individual enters his/her early twenties, commencing most often with the sagittal suture. Fusion of the coronal suture closure occurs in the mid-twenties and lastly the lambdoid suture in the late twenties (Morriss-Kay & Wilkie,
Thus, a failure in this sequencing resulting in the premature fusion of one or more of these sutures causes a birth defect known as craniosynostosis.

Figure 1: Major bones and sutures of the adult human cranium. Lateral (left) and top (right) view demonstrating the bones (red line) and sutures (blue) of the calvarium. The metopic suture separating the right and left halves of the frontal bone generally closes by the second year of life.

Reprinted with permission from Levi et al., 2012

1.2 Craniosynostosis terminology

Craniosynostosis is recognized clinically and radiographically as the premature fusion of one or more cranial sutures. It is the most common congenital defect of the human skull (Cohen, 1988; Robin et al., 2011). To aid in diagnosis and treatment, different classification systems are employed to describe the specific type of craniosynostosis. Classification is based upon the number of sutures involved, their anatomical locations, shape of the head, etiology, and whether the synostosis is an isolated physical difference or part of a syndrome.
Simple/complex and primary/secondary synostosis

Simple craniosynostosis is a term used to describe the premature fusion when only one suture fuses prematurely. When multiple sutures are involved, this is termed complex or compound craniosynostosis (Kimonis et al., 2007).

Primary synostosis results from a defect of ossification and may involve a single or multiple sutures, for example coronal synostosis only or coronal and sagittal synostosis (Cohen, 2002). It is believed that this premature fusion is the direct result of a developmental error during embryogenesis (Nagaraja et al., 2013). Secondary craniosynostosis is caused as a result of another medical condition or environmental issue, which subsequently impedes normal suture formation (Cohen, 2002; Kimonis et al., 2007). Numerous conditions are associated with secondary craniosynostosis, including metabolic disorders, hematological disorders and teratogens (Cohen & MacLean, 2000).

Syndromic craniosynostosis

When craniosynostosis is accompanied by other physical or developmental anomalies it is designated as syndromic craniosynostosis (Kimonis et al., 2007). All craniosynostosis syndromes have defects in common with each other as a result of the premature fusion of the cranial sutures. The shape of the skull is distorted due to the inability of growth to occur perpendicularly to the prematurely fused suture and the resultant supplementary overgrowth at the non-fused sutures of the cranium. In addition to craniosynostosis, several defects occur in the midfacial region such as midfacial hypoplasia, exophthalmos, dental problems, and orofacial clefting.

More than one hundred syndromes have been described in which craniosynostosis is a documented feature (Cohen, 2002; Cornejo-Roldan et al., 1999; Robin et al., 2011). Examples of craniosynostosis syndromes include Crouzon, Pfeiffer and Apert syndrome. These syndromes share characteristics such as midface hypoplasia, hypertelorism, exophthalmos, a hypoplastic maxilla and relative mandibular prognathism. Although similar in many respects, each syndrome differs, in particular in the extent of involvement of the limbs and the associated abnormalities that are observed in both Pfeiffer and Apert syndrome. Crouzon syndrome (CS) is the most common syndrome of the three and is generally the most mild in presentation with individuals
often having normal or near normal intelligence (Forrest & Hopper, 2013). CS is characterized by the absence of limb defects, in contrast to Pfeiffer and Apert syndromes (Johnson & Wilkie, 2011).

Pfeiffer syndrome (PS) is characterized by craniosynostosis of variable degrees, with distinct phenotypic features that include midface hypoplasia, severe exophthalmos, ocular hypertelorism, psittichorhina, broad radially deviated thumbs and toes, and in some cases partial cutaneous syndactyly of the hands and feet (Johnson & Wilkie, 2011; Lajeunie et al., 2006). Recognized central nervous system (CNS) malformations in PS include hydrocephalus and congenital or acquired Chiari malformation (Kabbani & Raghuveer, 2004; Ranger et al., 2010). Other recognized associations are radio-ulnar synostosis, and fusion of the cartilaginous tracheal rings (Akai et al., 2006; C.-P. Chen et al., 2008; Gonzales et al., 2005; Hockstein et al., 2004; Lajeunie et al., 2006; Oliveira et al., 2006; Ranger et al., 2010; Stevens & Roeder, 2006; Zackai et al., 2003). These severely affected patients frequently have a high mortality rate in infancy or early childhood (Akai et al., 2006; Lajeunie et al., 2006). PS is subdivided into three clinical types, 1, 2 and 3. Type 1 is a milder form of PS whereas both type 2 and 3 exhibit severe phenotypic expression. PS type 1 characteristics include craniosynostosis, broad thumbs and toes, and partial syndactyly of the hands and feet to varying degrees (Barone et al., 1993; Johnson & Wilkie, 2011; Koga et al., 2012). These individuals have normal or near normal intelligence (Barone et al., 1993). PS type 2 characteristics include craniosynostosis manifesting as a cloverleaf skull, broad thumbs and toes, severe ocular proptosis and central nervous system malformations. These individuals can also be affected with elbow ankylosis or synostosis and other anomalies (Barone et al., 1993). PS type 3 is very similar to type 2, but is differentiated by the lack of the cloverleaf skull. Further characteristics include shallow orbits, and a foreshortened anterior cranial base (Barone et al., 1993). Limb defects found in PS involves the broadening of the thumbs and/or big toes and may exhibit either unilateral or bilateral involvement. Some cases may also involve cutaneous syndactyly (Lajeunie et al., 2006). The severity and extent of involvement of the limbs is a useful diagnostic aid to distinguish between PS and Apert syndromes.

Apert syndrome (AS) is characterized by bicornoral craniosynostosis, midface hypoplasia and severe bilateral and symmetrical syndactyly of the hands and feet (Johnson & Wilkie, 2011; Kaplan, 1991; Wilkie et al., 1995). Another finding frequently found associated with AS is a
cleft palate (Johnson & Wilkie, 2011). Individuals with AS also have a high risk of learning impairments and disability (Johnson & Wilkie, 2011). The extent of the involvement and symmetry in the malformations of the hands and feet in AS distinguishes this syndrome from PS.

1.3 Incidence

The overall incidence of craniosynostosis has been estimated to range between 1:2100 to 1:3000 live births (Lajeunie et al., 1995). It has been estimated that approximately 85% of documented cases are isolated, involving only a single suture, with the remaining 15% of cases being syndromic (Chumas et al., 1997). The incidence of syndromic craniosynostosis is reported to be between one in 25,000 and one in 100,000 infants (Forrest et al., 2013).

The location of the synostosis, ranked from most to least commonly observed are the sagittal, coronal (either unilaterally or bilaterally), metopic, and then lambdoidal sutures (Cohen & MacLean, 2000; Johnson & Wilkie, 2011). Sagittal synostosis accounts for approximately 40-55% of non-syndromic synostotic cases, with coronal synostosis accounting for 20-25%, metopic synostosis accounting for 10-15% and finally lambdoidal synostosis occurring rarely up to 5% (Slater et al., 2008). Recent reports suggest that the involvement of the metopic suture may be more prevalent than previously reported (Forrest & Hopper, 2013). These studies indicate that metopic synostosis is found to in one in 10,000 live births compared to coronal synostosis occurring in one in 15,000 live births (Selber et al., 2008; van der Meulen et al., 2009). In general, there is a male predominance of the symmetric craniosynostoses (sagittal and metopic) and a female predominance of the asymmetric subtypes (Forrest & Hopper, 2013).

1.4 Management

The management of individuals with craniosynostosis can range from minimal involvement to requiring extensive care. Specifically, patients with syndromic craniosynostoses require complex care which is best approached by a multidisciplinary team to effectively address the vast array of their needs (Cunningham et al., 2007; Derderian & Seaward, 2012). Patients often require multiple staged procedures which are carried out throughout childhood and
adolescence at age-appropriate intervals (Forrest & Hopper, 2013). As an example, procedures may involve cranial vault remodeling, cleft repair, tracheostomies or limb reconstruction, depending upon the presenting problems. A multidisciplinary team should ideally include genetics, plastic surgery, neurosurgery, neuroradiology, dentistry, orthodontics, oral and maxillofacial surgery, ophthalmology, otolaryngology, anesthesiology, psychiatry, social work, occupational therapy, speech and language pathology and nursing in a tertiary care facility with expertise in this area (Forrest & Hopper, 2013). Expertise is established based upon volumes of patients and interest in the area (Cunningham et al., 2007).

1.5 Causes

The premature fusion of a suture or sutures may be the result of mechanical pressures, metabolic, or genetic defects or teratogenic exposure (Nagaraja et al., 2013). Intrauterine compression of the skull against the maternal pelvis could result in a mechanical cause of synostosis (Johnson & Wilkie, 2011; Kimonis et al., 2007; Levi et al., 2012; Oliveira et al., 2006). Hyperthyroidism is an example of a metabolic condition that can induce premature fusion (Kabbani & Raghuveer, 2004; Nagaraja et al., 2013). Numerous genetic factors have been implicated in the cause of synostosis. These genes encode proteins that are known to be involved in the control of intramembranous ossification of the skull (Coussens et al., 2007).

1.6 Genetic overview of craniosynostosis syndromes

The identification of specific genetic mutations, in both syndromic and non-syndromic cases of craniosynostosis, has led to a greater understanding of the etiology, development and presentation of these disorders.

In general, evidence supports an autosomal dominant mode of inheritance and it is believed that approximately 8% of all individuals with craniosynostosis have a dominant family history of this condition (Cohen, 2002). However, the phenotype is not always consistent among affected relatives of the same family. While some pedigrees do display fusion of a specific suture such as coronal, sagittal or metopic, others will exhibit synostosis of different sutures (Cohen,
Intrafamilial phenotypic variation can be extensive, leading to significant differences in management and morbidity.

Over 60 different mutations have been identified to be causal in syndromic forms of craniosynostosis. The majority of these mutations occur in the *FGFR2* gene. As part of a 10-year prospective cohort study testing 326 affected children, Wilkie and colleagues (2010) identified *FGFR2* as the most frequent mutation (32% of all genetic cases) followed by *FGFR3* (25%), *TWIST1* (19%) and *EFNB1* (7%). Other well established, but considerably rarer, associated genes include *FGFR1* (mild PS), *POR* (Antley-Bixler syndrome) and *RAB23* (Carpenter syndrome). Furthermore, other single-gene defects have been reported and include mutations in *EFNA4* (non-syndromic coronal synostosis), *ESCO2* (Roberts syndrome), *GLI3* (Greig syndrome), *JAG1* (Alagille syndrome), *KRAS* (Noonan syndrome), *RECQL4* (Baller Gerold syndrome), *TGFBR1* or *TGFBR2* (Loeys-Dietz syndrome) and *MSX2* (Boston type) (Florisson et al., 2013; Jabs et al., 1993; Johnson & Wilkie, 2011; Merrill et al., 2006; Wilkie et al., 2010).
2. FIBROBLAST GROWTH FACTOR (FGF) RECEPTORS

2.1 Structure

The activities of fibroblast growth factors (FGF) are mediated by four cell surface tyrosine kinase (TK) receptors, FGFR 1-4 (Ornitz & Marie, 2002). The receptors consist of an extracellular region, a single hydrophobic membrane-spanning segment and a cytoplasmic TK domain (Johnson et al, 1991). The extracellular area serves as the ligand recognition and binding site and it is composed of three immunoglobulin (Ig)-like domains, designated IgI–IgIII, a stretch of seven to eight acidic residues in the linker connecting IgI and IgII, designated the “acid box” and a conserved positively charged region in IgII that serves as a binding site for heparin (Schlessinger et al., 2000). Two highly conserved cysteine residues form an intramolecular disulfide bridge stabilizing the loop structure of the three Ig-like domains. The cytoplasmic domain contains the catalytic protein TK core which serves to recruit and activate docking and signaling proteins (Eswarakumar et al., 2005). Ligand binding to the FGFR in conjunction with a heparan sulfate proteoglycan (HSPG) causes receptor dimerization, transphosphorylation and activation of an intracellular TK domain (Jaye, Schlessinger, & Dionne, 1992). The dimeric FGFR is stabilized by both FGF-mediated interactions, direct receptor–receptor interactions as well as by binding of the HSPG to the heparin binding domains as well as the FGF molecules (Schlessinger et al., 2000). Receptor activation leads to the docking of adapter proteins followed by the activation of a signal transduction cascade that regulates genetic expression causing diverse biological responses (Szebenyi & Fallon, 1999). Receptors differ from one another mainly in their ligand affinities and tissue distribution (Burke et al., 1998).

2.2 Evolution

The genes coding for FGFs and their receptors have been identified in multicellular organisms ranging from the nematode, Caenorhabditis elegans, to the mouse, Mus musculus, and the human, Homo sapiens. However, they have not been identified in unicellular organisms such as Escherichia coli and Saccharomyces cerevisiae. Evidence exists indicating that both the FGF and FGFR gene families have greatly expanded during the evolution of primitive metazoa to vertebrates. The co-evolution of FGF and FGFR genes has permitted increased ligand-receptor
specificity, enabling the formation of preferred ligand-receptor interactions offering this signaling system a great functional diversity and therefore an almost ubiquitous involvement in developmental and physiologic processes (Itoh & Ornitz, 2004).

2.3 Biological roles

In recent years, the analysis of FGFR mutations involved in human skeletal disorders and advances in mouse genetics have revealed essential molecular mechanisms by which FGF/FGFR signaling controls tissue formation. Reported roles of FGFRs have included regulation of cell division, cell growth and maturation, formation of blood vessels, wound healing and bone growth during embryonic development (Hughes, 1997). FGFRs have been intensely studied as a possible anticancer target for their role in tumour cell proliferation, angiogenesis and migration (Kumar, Narasu, Gundla, Dayam, & J A R P, 2013) and for their involvement in bone formation and metabolism (Du et al., 2012).

In the skeleton, FGF/FGFR signaling is an important regulator of prenatal and postnatal bone development (Marie, 2003; Ornitz & Marie, 2002). During embryonic development, the FGF/FGFR interaction has been shown to control the switch between adipocyte and osteoblast differentiation in mesenchymal bone marrow stromal cells (Xiao et al., 2010). Genetic studies in mice and humans have highlighted the important role of FGFR1/2 signaling in the control of osteoblast gene expression and bone formation (Wilkie, 2005). FGF2 signaling activates many internal signaling pathways in osteoblasts, including mitogen-activated protein (MAP), extracellular signal-regulated kinases (ERKs), p38 MAP kinase and protein kinase C (PKC). These pathways were known to mediate osteoblast proliferation, expression of specific osteoblast genes and survival (Marie, 2003).

2.4 Mutations of FGFRs in craniosynostosis syndromes

Mutations of FGFR1-3 have been associated with a variety of phenotypic abnormalities and often significant phenotypic variability is observed even among familial cases (Passos-Bueno et al., 1999). Few phenotype-genotype correlations are absolute suggesting that
phenotypic features may also be determined by epigenetic factors or unlinked modifier genes (Robin et al., 2011).

What is known is that the majority of FGFR mutations act in an autosomal dominant way, are frequently de novo and result in increased activation of the mutant receptor, sometimes independently of the ligand (Passos-Bueno et al., 1999). This is termed a “gain-of-function” mutation.

Missense or splice-site mutations account for the majority of all observed nucleotide changes with the occasional occurrence of small inframe insertions and deletions being documented in FGFR-associated craniosynostosis syndromes (Cohen, 2002). FGFR mutations may affect the extracellular or intracellular domains (Cohen & MacLean, 2000). When FGFR mutations create or destroy cysteine residues, an unpaired cysteine remains that can generate intermolecular disulfide bonding resulting in constitutive receptor activation (Neilson & Friesel, 1996). These gain of functions mutations cause alterations that can cause varying degrees of ligand-independent signaling. An FGFR mutation can allow early human development to proceed normally but may exert its interference with later bone development (Cohen, 2002).

2.5 Genotype-phenotype correlations

Missense mutations in FGFR1-3 have been associated with craniosynostosis and human chondrodysplasia (Table 1). Typically, these mutations account for eight predominant syndromes, comprising the FGFR-related craniosynostosis spectrum (Robin et al., 2011). These include: AS, PS, CS, CS with acanthosis nigricans (CS-AN), Beare-Stevenson syndrome, FGFR2-related isolated coronal synostosis, Jackson-Weiss syndrome and Muenke syndrome. All of these mutations are autosomal dominant and often arise spontaneously (Ornitz & Marie, 2002).
Table 1: FGFR mutations and associated disorders.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Characteristic mutation (% responsible)</th>
<th>Disorder</th>
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<tbody>
<tr>
<td><strong>FGFR1</strong></td>
<td>Widespread (haploinsufficiency) P252R (100%), N330I, Y374C, C381R</td>
<td>Kallman syndrome, Pfeiffer syndrome (PS), Osteoglophonic dysplasia</td>
</tr>
<tr>
<td></td>
<td>S252W (66%), P253R (32%), IgIIIC (71%), IgIIIA (23%), TK, IgI</td>
<td>Apert syndrome (AS), Crouzon(CS)/Pfeiffer(PS)/Jackson-Weiss/Antley-Bixler syndromes, Beare-Stevenson syndrome</td>
</tr>
<tr>
<td></td>
<td>Y375C (&gt;85%), S372C</td>
<td></td>
</tr>
<tr>
<td><strong>FGFR2</strong></td>
<td>P250R (&gt;99%), A391E (100%) G380R (99%), K650M (100%) N540K (42%), R248C (56%), Y373C (22%), stop codon (12%), K650E (100%)</td>
<td>Meunke syndrome, Crouzon syndrome with acanthosis nigricans(CS-AN), Achondroplasia, Severe achondroplasia with acanthosis nigricans, Hypochondroplasia, Thanatophoric dysplasia I (TDI), Thanatophoric dysplasia II (TDII)</td>
</tr>
</tbody>
</table>

Adapted with permission from Wilkie, 2005

Three distinct craniosynostosis syndromes (CS, PS and AS) are usually caused by heterozygous mutations of FGFR2 (Johnson & Wilkie, 2011). The mutations can be widely distributed across the FGFR2 gene, yet the majority are found in the IgIIIA/IIIC domain. Several critical mutational “hot spots” have been described in this domain for CS and PS patients in codons 278, 290 and 342 (Kress et al., 2000; Schaefer et al., 1998). Mutations in these areas can result in unpaired cysteine residues, ultimately disrupting the protein structure by forming intermolecular dimerization that leads to constitutive activation of the receptor (Neilson & Friesel, 1996).

Interestingly, mutations occurring on the same codon can exhibit very different phenotypes as a result of which nucleotide substitution occurs. For example, two FGFR2 mutations creating cysteine residues (p.Trp290Cys and p.Tyr340Cys) cause severe forms of PS whereas conversion of the same residues into another amino acid (p.Trp290Gly, p.Trp290Arg, p.Tyr340His) results exclusively in the CS phenotype (Lajeunie et al., 2006). This suggests that alteration of the protein may cause a different functionality of the gene itself.
Figure 2: Distribution of germline mutations in FGFR1, FGFR2 and FGFR3 causing missense substitutions (above protein) or probable splicing abnormalities (below protein). The mutation prevalence is indicated on a logarithmic scale and different phenotypes are color-coded. Where multiple phenotypes occur with mutation at a single position, the column colors are partitioned proportionately using a linear scale. Substitutions recorded in more than 20 independent subjects.
are identified individually. For clarity, insertions, deletions and nonsense mutations (none of which has occurred as a recurrent mutation) are omitted; “Antley–Bixler” and “Jackson–Weiss” phenotypes are lumped with Pfeiffer syndrome. Data for FGFR1 and FGFR2 are from Wilkie’s unpublished database; data for FGFR3 are from Passos-Bueno et al. (1999), supplemented with additional mutations. In each case the alternatively spliced IIIc spliceform is shown; no mutations have been described in any of the IIIb exons.

Reprinted with permission from Wilkie, 2005

More than 50 mutations have been described in CS with approximately 95% of them being located in just two exons of the gene encoding the extracellular IgIII domain, i.e., the IIIa and IIIc domains (Meyers et al., 1996). Many of these mutations are believed to alter downstream receptor signaling (Anderson et al., 1998; Ibrahimi et al., 2001; Mangasarian et al., 1997; Mansukhani et al., 2000; Plotnikov et al., 2000). Mutations causing AS have been shown to result in the loss of ligand binding specificity and thus can be activated by inappropriate ligands, whereas CS and PS mutations in FGFR2 result in ligand independent activation and dramatic decreased ligand binding (Ibrahimi et al., 2001; Mangasarian et al., 1997; Marie et al., 2012; Plotnikov et al., 2000).

The mutations associated with PS overlap considerably with that of CS. PS type 1 can be caused by a mutation in FGFR1 or FGFR2 (Koga et al., 2012). PS type 2 and 3 manifest as more severe forms of the disorder, both occurring as de novo mutations. A small subset of substitutions in the FGFR2 gene encoding p.Trp290Cys, p.Try340Cys, p.Cys342Arg or p.Ser351Cys account for a large majority of the severely affected cases (Johnson & Wilkie, 2011).

Over 98% of patients diagnosed with AS have been found to have a missense mutation of FGFR2 in the linker between the IgII and IgIII domains at codon p.Ser252Trp (66%) or p.Pro253Arg (32%) (Johnson & Wilkie, 2011). In these cases some phenotypic correlations can be drawn depending upon the specific mutation. Individuals with the FGFR2 mutation p.Ser252Trp tend to present more often with cleft palates, severe ocular problems and nasolacrimal stenosis (Akai et al., 2006). Intellectual disability and syndactyly appears to be
more severe in individuals with the p.Pro253Arg mutation in the \textit{FGFR2} gene (Akai et al., 2006; Jadico et al., 2006; Johnson & Wilkie, 2011; Slaney et al., 1996).

Mutations in \textit{FGFR3} are involved in both craniosynostosis and human chondrodysplasias. A correlation has been observed between a mutation in \textit{FGFR3} (p.Ala391Glu) and the development of acanthosis nigricans (AN) in patients with CS; however, patients with CS without AN are very unlikely to display this specific mutation (Robin et al., 2011). Achondroplasia, the most common form of short-limb dwarfism, has been linked to specific mutations within the transmembrane region of \textit{FGFR3} with G380R accounting for nearly all cases (Rousseau et al., 1996; Shiang et al., 1994; Wilkie, 2005)
3. GASTROINTESTINAL TRACT (GIT)

3.1 GI development and FGF signaling

The molecular basis for the development of the GIT is not completely understood. Morphogens, substances that originate from a localized source and form a concentration gradient throughout a tissue, are involved in the building of complex organs with intricate patterns of cellular specialization (Wolpert, 1996). A few highly conserved morphogenetic signaling pathways have now been identified in the development of the GIT. The four most important morphogenetic signaling pathways that have been shown to play a role in patterning of the early gut tube are FGF, Hedgehog, Wnt and Transforming Growth Factor (TGF)-β. These pathways are conserved among organisms from the fruit fly to the human (Dab et al., 2013; van den Brink, 2007).

At a molecular level, studies have demonstrated that the FGFRs and the FGF ligands are expressed along the entire GIT and are known to play a critical role in the development of GI structures and multiple other organ systems. High levels of expression of FGFR2 have been found throughout the GIT in adult human tissues (Hughes, 1997), specifically throughout the gastric epithelium and submucosal macro- and microvasculature, with marked expression in the muscularis mucosae and muscularis. FGFR2 expression is widespread in the entire mucosal epithelium and muscularis in the entire ileum and appendix, with minimal expression observed in hepatocytes and bile duct (Hughes, 1997).

Mouse models have been instrumental in uncovering many of the potential abnormalities of the GIT associated with Fgfr2 mutations. Fairbanks et al., (2004) reported that duodenal atresia was present with a 35% penetrance in Fgfr2b mutant mice embryos. A CS mouse model with a specific Fgfr2 mutation (in codon 290 of Fgfr2), a mutation also found in humans with CS, has been characterized to present with numerous GI defects (Dab et al., 2013; Mai et al., 2010). The Fgfr2<sup>W290R</sup> mutants had numerous defective organ formation such as bones, teeth, glands, and lungs (Mai et al., 2010). Additionally, they also presented with specific histological, structural and functional defects in the esophagus (Dab et al., 2013). The GIT were much shorter with a relatively empty peritoneal cavity. The mutant mice exhibited a decreased thickness in the both the muscle layers and epithelium of the esophagus with a reduction in cell proliferation noted. The esophageal layers also contained reduced collagen contention in
conjunction with a distinct patterning change in the orientation of muscle fibers of these mutant mice and demonstrated reduced contractile activity compared to wildtype mice (Dab et al., 2013). The findings in the \( Fgfr2^{W290R} \) mutant mice therefore suggest a strong correlation between the \( Fgfr \) mutation and GI malformations. The correlation therefore lends a plausible scientific explanation for the anecdotal and observed findings of GI problems in \( Fgfr2 \) related craniosynostosis patients, such as CS.

3.2 GI malformations

The GIT system is complex both developmentally and structurally. During embryonic development, abnormalities of development of the GIT may involve one or multiple areas with or without involvement of the other systems in the body. Some anomalies are readily detected, yet others may not initially be apparent.

Common congenital GI malformations found in patients treated at a tertiary/quaternary care academic hospital, the Hospital for Sick Children (SickKids) in Toronto, Ontario, Canada, include esophageal atresia with or without tracheo-esophageal fistula, intestinal atresia, malrotation with volvulus, Hirschsprung's disease, ano-rectal malformations and abdominal wall defects. These anomalies are often classified using the European Surveillance of Congenital Anomalies (EUROCAT) system.

**EUROCAT classification system**

EUROCAT is a network of population-based registries for the epidemiologic surveillance of congenital anomalies. This database is useful for providing incidence data for rare congenital anomalies, including those of the digestive system and abdominal wall defects. This registry has documented 1.7 million births in 21 countries of Europe. ([http://www.eurocat-network.eu/](http://www.eurocat-network.eu/)).

Categories classified under the EUROCAT heading of Digestive System include: oesophageal atresia with or without tracheo-oesophageal fistula, duodenal atresia or stenosis, atresia or stenosis of other parts of small intestine, ano-rectal atresia and stenosis, Hirschsprung’s disease, atresia of bile ducts, annular pancreas, diaphragmatic hernia. Additional categories to be reviewed included intestinal malrotation and gastroesophageal reflux disease (GERD).
Categories classified under the EUROCAT heading of abdominal wall defects include gastroschisis and omphalocele. A description of each anomaly is provided in the Table 2 below.

### Table 2: EUROCAT category and description of gastrointestinal anomaly.

<table>
<thead>
<tr>
<th>Digestive system</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oesophageal atresia with or without tracheo-oesophageal fistula</td>
<td>Occlusion or narrowing of the oesophagus with or without tracheo-oesophageal fistula</td>
</tr>
<tr>
<td>Duodenal atresia or stenosis</td>
<td>Occlusion or narrowing of duodenum</td>
</tr>
<tr>
<td>Atresia or stenosis of other parts of small intestine</td>
<td>Occlusion or narrowing of other parts of the small intestine</td>
</tr>
<tr>
<td>Ano-rectal atresia and stenosis</td>
<td>Imperforate anus or absence or narrowing of the communication canal between the rectum and anus with or without fistula to neighbouring organs</td>
</tr>
<tr>
<td>Hirschsprung’s disease</td>
<td>Absence of the parasympathetic ganglion nerve cells (aganglionosis) of the wall of the colon or rectum. May result in cong. megacolon.</td>
</tr>
<tr>
<td>Atresia of bile ducts</td>
<td>Congenital absence of the lumen of the extrahepatic bile ducts</td>
</tr>
<tr>
<td>Annular pancreas</td>
<td>Pancreas surrounds the duodenum causing stenosis</td>
</tr>
<tr>
<td>Diaphragmatic hernia</td>
<td>Defect in the diaphragm with protrusion of abdominal content into the thoracic cavity. Various degree of lung hypoplasia on the affected side</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Abdominal wall defects</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastroschisis</td>
<td>Protrusion of abdominal contents through an abdominal wall defect lateral to an intact umbilical cord and not covered by a membrane</td>
</tr>
<tr>
<td>Omphalocele</td>
<td>Herniation of abdominal content through the umbilical ring, the contents being covered by a membrane sometimes at the time of delivery.</td>
</tr>
</tbody>
</table>

Source: European Surveillance of Congenital Anomalies (http://www.eurocat-network.eu/)
**Intestinal malrotation**

Intestinal malrotation is a serious and potentially fatal developmental anomaly. Both the small and large intestines can be affected during development in fetal life involving both the position and peritoneal attachments leading to malrotation (Cassart et al., 2006). In majority of cases, diagnosis is made during the first year of life (Pickhardt & Bhalla, 2002). Diagnostic symptoms include abdominal pain, bile stained vomit secondary to bowel obstruction, bowel dilatation, ascites or meconium peritonitis (Nehra & Goldstein, 2011). Nevertheless, the true incidence of intestinal malrotation remains unknown (Nehra & Goldstein, 2011). The literature cites incidences ranging from 1:200 to 1:6000, with an incidence of 1:500 being commonly referenced (Cassart et al., 2006; Nehra & Goldstein, 2011; Pickhardt & Bhalla, 2002).

**Gastroesophageal Reflux Disease (GERD)**

GERD is a very common disorder of the GIT (Bredenoord, Pandolfino, & Smout, 2013). It has been defined as a chronic condition developing due to significant quantities of reflux of gastric contents into the esophagus causing symptoms with or without mucosal erosions and/or associated complications (Chen & Hsu, 2013).

The true incidence of GERD in the general population is difficult to discern, but can be classified in terms of its prevalence. In the adult population, GERD can be defined by at least weekly heartburn and/or acid regurgitation, with prevalence estimates ranging from 10 to 20% in western countries and less than 5% in Asian countries (Dent et al., 2005; El-Serag et al., 2009; Moayyedi & Talley, 2006). The estimated prevalence of GERD in infants is considered to have a peak incidence of approximately 50% at 4 months of age and then to decline affecting only 5% to 10% of infants at 12 months of age (Lightdale & Gremse, 2013). Vomiting will settle in majority of infants by 13 or 14 months of age (A. Martin et al., 2002). While this is merely an estimate, the reported prevalence of GERD in patients of all ages worldwide is increasing (Sherman et al., 2009). Diagnosis of GERD in infants and children can be difficult and is usually made based upon presenting symptoms. In infants, common symptoms include regurgitation or vomiting associated with irritability, anorexia or feeding refusal, poor weight gain, dysphagia, presumably painful swallowing, and arching of the back during feedings. In young children, aged 1 to 5 years old, common symptoms of GERD include regurgitation, vomiting, abdominal pain, anorexia, and feeding refusal (Gupta et al., 2006). Older children and adolescents are likely to
resemble a similar clinical presentation to adults with GERD including symptoms such as heartburn, epigastric pain, chest pain, nocturnal pain, dysphagia, and unpleasant tasting burps (Lightdale & Gremse, 2013). The two typical symptoms of GERD are recognized as pyrosis, commonly referred to as heartburn and regurgitation, with some patients also noting extraesophageal symptoms such as hoarseness, cough, and asthma (Bredenoord et al., 2013). There is no gold standard test for objectively diagnosing GERD, and thus diagnoses have relied on a combination of disease characteristics (Bredenoord et al., 2013).

3.3 Craniosynostosis and structural/functional anomalies of the GI system

Children with craniosynostosis syndromes may have other associated health problems including GI malformations (Cohen & Kreiborg, 1993). Interestingly, mutations in FGFR2, the most common gene associated with craniosynostosis syndromes have also been linked to case reports of GI disorders. The literature on this topic is surprisingly sparse; however, there are few studies and several of case reports on the subject.

Cohen and Kreiborg (1993) examined 135 patients with AS and concluded that only 1.5% presented with GI problems compared to 10% affected with cardiovascular anomalies, 9.6% with genitourinary anomalies 9.6%, and 1.5% with respiratory anomalies. Notably, when autopsies were performed on 12 of these 135 patients, visceral anomalies including congenital heart defects, anomalies of the respiratory system, GIT anomalies and genitourinary abnormalities, were found in 9 of 12. This suggests that the percentage of anomalies from clinical examination and history should be considered the minimum estimate because of the possibility of clinically silent visceral anomalies, minor internal anomalies and anatomic variations.

A recent study by Koga et al. (2012) investigated the clinical expression of PS types 2 and 3 in a Japanese population. Records of a total of 23 patients with confirmed PS diagnosis, treated in one Japanese hospital between 1980-2011, were examined. Striking findings included the detection of GI malformations in 22% of these patients. The detected problems included an imperforate anus, intestinal malrotation and intestinal atresia in the affected individuals (Koga et al., 2012).
Only a handful of case reports are cited in the literature ranging from 1975 to the present documenting craniosynostosis patients who also presented with GI problems. In 1975, Eaton and colleagues identified a patient with PS with features suggestive of type 2, who was found to have intestinal malrotation. Barone et al, (1993) examined two patients with PS type 3, who developed bowel obstruction and were also found to have intestinal malrotation. Park et al., (1995) identified a mother and son both with CS and both diagnosed with epithelial- derived anal atresia. More recently, Zarate and colleagues (2010) reported a case of a 16 month old male with craniosynostosis who underwent cranial vault remodeling, placement of VP shunts for hydrocephalus, gastrostomy tube for suspected GERD, tracheostomy due to bilateral choanal stenosis, craniofacial reconstruction and posterior fossa decompression. The diagnosis of GI malformation was very delayed resulting in significant morbidity including numerous infections and respiratory problems. In another recent unpublished case report (2010) from the Hospital for Sick Children (SickKids) in Toronto, an infant presented with severe bicoronal synostosis and a diagnosis of PS, who subsequently presented with symptoms of a small bowel obstruction, secondary to volvulus and a duodenal web.

These documented case reports have indicated the possibility that GI malformations could be a direct effect of the FGFR gene mutation associated with craniosynostosis, a possibility further supported by research in animal models. Specifically, all the discussed case reports have a common finding of intestinal rotation. We therefore propose that a correlation between FGFR-associated craniosynostosis and GIT malformations exists.
II. HYPOTHESIS AND OBJECTIVES

Hypothesis

We hypothesized that FGFR-related craniosynostosis is associated with GI malformations at a greater incidence than the general population.

Objective

The aim of this study was to determine whether GI malformations occur more frequently in the craniosynostosis population with a known \textit{FGFR} mutation compared to the general population.
A retrospective chart review of patients diagnosed with CS, PS or AS at the Hospital for Sick Children (SickKids) between 1990 and 2011 was performed. Approval was obtained from the Research Ethics Board at SickKids (Appendix A & B),

Inclusion criteria of charts to be included for review included the following:

- a) Record of genetic counseling, testing or discussion.
- b) Diagnosis of a craniosynostosis syndrome (CS, PS or AS)
- c) Identification of a mutation in FGFR1, 2 or 3 genes
- d) Documentation of a GI malformation according to the EUROCAT classification system and common congenital GI malformations seen at SickKids

Exclusion criteria included:

- a) Incomplete medical records
- b) Lack of documented evidence of mutations in FGFR1, 2 or 3.

Three databases were searched for patients meeting the study inclusion criterion:

- i) Division of Clinical and Metabolic Genetics clinical database (SHIRE systems)
- ii) Department of Paediatric Laboratory Medicine molecular genetics database (SHIRE systems), and
- iii) Division of Plastic Surgery craniofacial database.

Medical records of the selected patients treated at SickKids included review of molecular genetic test reports, general clinic letters, consultations, diagnostic imaging reports, clinic notes for plastic surgery, genetics and GI visits and emergency records. These charts were reviewed using a systematic approach that included a step-by-step analysis of all the records contained in each patient folder to assess for a definitive diagnosis of craniosynostosis, the presence of mutations in FGFR1, 2 or 3 genes, and any record of gastrointestinal abnormalities. If GIT abnormalities were noted, they were classified using the EUROCAT classification system (http://www.eurocat-network.eu/) for the following GIT anomalies:

- i) oesophageal atresia with or without tracheo-oesophageal fistula
- ii) intestinal malrotation, stenosis or atresia,
iii) GERD
iv) ano-rectal atresia or stenosis
v) Hirschsprung's disease,
vi) atresia of the bile ducts
vii) annular pancreas,
viii) diaphragmatic hernia,
ix) gastroschisis,
x) omphalocele

In addition, the investigation was further expanded to include findings of clefts of the palate and/or lip. Findings were documented in a coded spreadsheet to preserve patient anonymity.

**Statistical analysis:**

Statistical analysis was carried out by an independent statistician. A power calculation and a binomial test was conducted to establish the statistical significance of the deviation in the proportion of patients with GIT malformations in this sample compared to that of the general population, at the 0.05 significance level.
IV. RESULTS

1. Patient sample:

A total of 32 patient charts were identified that met all the inclusion criteria of the study. An initial review of the three databases of patients treated at SickKids between 1990 and 2011 identified a total of 50 charts of patients with clinical diagnoses of CS, PS and AS. As SickKids is a hospital for children, all patients were 18 years of age or younger. Out of the 50 charts identified initially, 18 patients were excluded from the study: 5 were due to inadequate health records, 8 to a lack of documentation of genetic testing for FGFR1, 2 or 3, and 5 due to the documented absence of a detectable FGFR mutation.

The 32 patient charts were reviewed for the congenital GIT malformations as described in the EUROCAT classification system and/or frequently encountered at SickKids.

2. Craniosynostosis syndromes and mutations:

Of the 32 patients that met the inclusion criteria, 10 were diagnosed with CS, 7 with PS and 15 with AS (Tables 3 and 4). All 32 patients were found to have documented FGFR2 mutations. Information on the specific mutations was only available for 25 patients. In total, 16 different mutations were identified in the 25 patients (Table 4 and Figure 3).

Of the 10 patients diagnosed with CS, all mutations were missense mutations in the IgIII domain of FGFR2. Three out of the 10 mutations were in codon 342, with the change of the cysteine (Cys) residue to tyrosine (Tyr), phenylalanine (Phe) and serine (Ser). The other mutations included changes in codons 290, 328, and 344.

PS patients in the sample contained missense mutations in the IgIII domain, and in the linker region between IgII and IgIII (S252) of FGFR2. In two of these patients, there was a change in codon 342 from Cys to arginine (Arg). A splice-site mutation was identified in the IgIII domain of FGFR2.
Of the 15 patients diagnosed with AS, 4 lacked documentation regarding the specific FGFR2 mutation. One patient was found to have a large intronic deletion of 1372 base pairs (bp) between FGFR2 exons IIIb and IIc. The remaining 10 patients had missense mutations in the linker region between IgII and IgIII in either Ser252 or proline (Pro)253. Seven of these 10 patients presented with a mutation at codon 252 from Ser to tryptophan (Trp), while three patients presented with a mutation at codon 253 from Pro to Arg.

**Table 3:** Number and type of mutations identified in a sample of 32 FGFR-associated craniosynostosis syndromic patients at the Hospital for Sick Children (Toronto, Ontario) between the years of 1990-2011.
**Table 4:** Observed Fibroblast Growth Factor Receptor mutations and associated findings. IM - intestinal (bowel) malrotation; GERD - gastroesophageal reflux disease; CP - cleft palate; CL - cleft lip; * likely reporting error in mutation nomenclature.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Syndrome</th>
<th>Gene</th>
<th>Mutation</th>
<th>Associated Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apert</td>
<td>FGFR2</td>
<td>p.Ser252Trp</td>
<td>CP</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>FGFR2</td>
<td>p.Ser252Trp</td>
<td>CP</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>FGFR2</td>
<td>specific mutation not available</td>
<td>GERD, CP</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>FGFR2</td>
<td>p.Ser252Trp</td>
<td>CP</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>FGFR2</td>
<td>p.Ser252Trp</td>
<td>CP</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>FGFR2</td>
<td>p.Pro253Arg</td>
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<tr>
<td>7</td>
<td></td>
<td>FGFR2</td>
<td>p.Pro253Arg</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>FGFR2</td>
<td>p.Pro253Arg</td>
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</tr>
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<td></td>
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<td>p.Ser252Trp</td>
<td>IM, GERD, CP</td>
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<td>CP</td>
</tr>
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</tr>
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<td>CP</td>
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<td>15</td>
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</tr>
<tr>
<td>16</td>
<td>Pfeiffer</td>
<td>FGFR2</td>
<td>p.Cys342Arg</td>
<td>IM, GERD</td>
</tr>
<tr>
<td>17</td>
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<td>FGFR2</td>
<td>c.856A&gt;T *</td>
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<td>25</td>
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<td>FGFR2</td>
<td>p.Cys342Phe</td>
<td>GERD</td>
</tr>
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<td>FGFR2</td>
<td>p.Ala344Ala</td>
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</tr>
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<td></td>
<td>FGFR2</td>
<td>p.Tyr328Cys</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>FGFR2</td>
<td>p.Cys342Ser</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>FGFR2</td>
<td>exon IIIa T880C *</td>
<td>CP, CL</td>
</tr>
<tr>
<td>31</td>
<td></td>
<td>FGFR2</td>
<td>exon IIIa T880C *</td>
<td>CP, CL</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>FGFR2</td>
<td>specific mutation not available</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3: Distribution and types of mutations observed in the sample population of CS, PS and AS patients (pictured above FGFR2 protein).

3. GIT anomalies

The review of the charts for GIT abnormalities according to the EUROCAT classification system revealed a relative absence of documentation of most GI problems. The most common abnormalities observed were intestinal/bowel malrotation (IM) and GERD.

Three out of 32 patients were found to have IM, and also exhibited GERD (Table 4). Of the three patients with IM, two of them were diagnosed with PS, with the mutation cysteine to arginine in codon 342. The third patient was diagnosed with AS with a mutation in codon 252 with a change from serine to tryptophan.
**Table 5**: Frequency of Intestinal/Bowel Malrotation and GERD in the sample population of CS, PS and AS patients.

<table>
<thead>
<tr>
<th></th>
<th>Sample Size</th>
<th>GERD</th>
<th>( % of total)</th>
<th>Malrotation</th>
<th>( % of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apert</strong></td>
<td>15</td>
<td>3</td>
<td>9.375</td>
<td>1</td>
<td>3.13</td>
</tr>
<tr>
<td><strong>Pfeiffer</strong></td>
<td>7</td>
<td>2</td>
<td>6.25</td>
<td>2</td>
<td>6.25</td>
</tr>
<tr>
<td><strong>Crouzon</strong></td>
<td>10</td>
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<td>6.25</td>
<td>0</td>
<td>0</td>
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<tr>
<td><strong>Total</strong></td>
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<td>7</td>
<td>21.875</td>
<td>3</td>
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The incidence in the general population of IM is 1 in 500 ($p_{genral}=0.002$) (Cassart et al., 2006; Nehra & Goldstein, 2011; Pickhardt & Bhalla, 2002). Statistical analysis comparing the incidence of IM in this sample population revealed a significantly higher incidence of this GI abnormality compared to the general population ($p < 0.05$), thus we conclude that the proportion with intestinal malrotation in our sample is greater than that of the general population.

Seven out of 32 patients were found to have GERD (Table 5). Three were AS patients, two were PS and one was a CS patient. No apparent pattern of genetic mutations is evident. The true incidence of GERD in the general population is very difficult to discern and estimated at 10-20% in western countries (Dent et al., 2005; El-Serag et al., 2009; Moayyedi & Talley, 2006). The incidence of 21.75% (7/32) in the sample population appeared to be similar to the incidence found in the general population in western countries.

Cleft palate (CP) was identified in a total of 12 cases. Nine cases were found in patients with AS, 1 in a patient with PS and two in patients with CS. A total of seven cases with CP displayed a mutation in codon 252 with a change from serine to tryptophan, while one case of CP was found in a patient with a mutational change from cysteine to arginine in codon 342. The remaining four cases identified with CP did not have documentation available of specific mutation involved.
4. Summary

In summary, the major findings of our study are:

1. The sample of 32 patient charts reveals 16 different genetic mutations in the \textit{FGFR2} gene.

2. Mutations in CS patients were found in the IgIII domains, mutations in PS patients occurred in both the IgIII domain, and in the linker region between IgII and IgIII (Ser252) of FGFR2, and mutations in AS patients were found in the linker region between IgII and IgIII in either Ser252 or Pro253.

3. The incidence of IM was significantly higher in the sample population compared to the incidence reported in the general population.

4. The incidence of CP was found almost exclusively in AS involving the p.Ser252Trp mutation.
V. DISCUSSION

This study showed a statistically significant association between FGFR-associated craniosynostosis and specific GI defects. To our knowledge, no studies to date have examined this population to look for this specific morbidity involving the GIT. Our study addresses this by reviewing the hospital records of a group of FGFR-associated craniosynostosis syndromic patients for the occurrence of GIT malformations.

1. Genetic findings in sample population

The current study found a total of 16 different genetic mutations in the 32 patient charts reviewed. Generally, the mutations observed in our cohort are similar to those previously documented in the literature. There has been quite extensive documentation of mutations in FGFR2 at codon 342, as well as 290 and 278 in both CS and PS patients and are regarded as mutational hotspots (Schaefer et al., 1998; Kress et al., 2001). The literature shows that mutations at codon 342, such as p.Cys342Arg and p.Cys342Trp tend to occur most frequently on the FGFR2 gene (Kress et al., 2001). Other commonly found mutations in individuals with CS and PS include p.Trp290Gly, p.Trp290Arg, p.Tyr340His and p.Cys278Phe mutation (Lajeunie et al., 2006; Kress et al., 2001, Park et al., 1995). Indeed, this was also the observation made in our study. Codon 342 appeared to be the most common codon affected in the sample population. Mutational changes of the cysteine residue to either Tyr, Phe or Ser were found in three CS patients and to Cys or Arg in two PS patients. Further, a mutation at codon 290 from Trp to Gly was observed in a CS patient. In addition to the missense mutations noted, one PS patient in our cohort sample exhibited a splice site mutation at 952-1G>A. The mutation of this patient has been previously described by Teebi and colleagues (2002).

In our study, two patients exhibiting intestinal malrotation were found to have the p.Cys342Arg mutation in the FGFR2 gene. This particular mutation, along with p.Ser351Cys, p.Trp290Cys has been detected in the more severe phenotypic displays of PS (Johnson & Wilkie, 2011).
Previous studies have reported that 98% of AS cases can be accounted for by two mutations 1) p.Ser252Trp and 2) p.Pro253Arg (Ferreira et al., 1999; Johnson & Wilkie, 2011). Literature suggests that the p.Ser252Trp mutation accounts for approximately two-thirds of the AS cases and the p.Pro253Arg mutation for the other third (Ferreira et al., 1999; Johnson & Wilkie, 2011). Similar proportions were observed in our study, with the p.Ser252Trp mutation occurring more often than the p.Pro253Arg mutation. Phenotype-genotype correlations for these specific mutations have been previously documented. Individuals with an FGFR2 mutation of p.Ser252Trp tend to present more often with cleft palates, severe ocular problems and nasolacrimal stenosis (Akai et al., 2006). Patients with p.Pro253Arg in the FGFR2 gene often display more intellectual disability and greater degrees of syndactyly (Akai et al., 2006; Jadico et al., 2006; Johnson & Wilkie, 2011; Slaney et al., 1996). The sample population in our study also supports these previously documented findings with six out of seven individuals with p.Ser252Trp mutation displaying cleft palates. The AS patient exhibiting a large intronic deletion was found to harbour a heterozygous 1372 bp deletion between FGFR2 exons IIIb and IIIc, This apparently originated from recombination between 13 bp of identical DNA sequence present in both exons and was not found in the unaffected parents (Fenwick et al., 2011).

2. Intestinal malrotation in sample population

Our findings suggest an association between FGFR-related craniosynostosis and GI malformations, in particular bowel malrotation. The results show a statistically significant difference in the number of FGFR-associated craniosynostosis patients that present with an intestinal (bowel) malrotation when compared to the normal population. Patients in our cohort are approximately 47 times more likely to have bowel malrotation. These results support our hypothesis that FGFR-related craniosynostosis is associated with GI malformations. These findings are not surprising given what is already known about FGFR involvement in normal growth and development. They are further supported by multiple mouse model experiments, case reports and clinical studies, revealing GIT anomalies in the syndromic craniosynostosis populations (Barone et al., 1993; Cohen & Kreiborg, 1993; Eaton et al., 1975; Fairbanks et al., 2004; Gong, 2012; Johnson & Wilkie, 2011; Lajeunie et al., 1999; Mai et al., 2010; Oldridge et
al., 1997; Park et al., 1995; Passos-Bueno et al., 1999; Wilkie, 2001; Wilkie et al., 1995; Zarate et al., 2010).

Several studies examining phenotypic expression in the human FGFR-associated craniosynostosis populations similarly point towards such correlations. A retrospective study by Koga and colleagues (2012), carried out in Japan, has cited repeated malformations in the GIT as part of their findings. In their sample population, 22% of the PS patients exhibited GIT malformations including an imperforate anus, and intestinal malrotation (Koga et al., 2012). Our study showed similar results with 25% of PS patients presenting with some type of GIT malformation. Cohen and Kreiborg’s, (1993) study thoroughly examined 136 AS cases and concluded that 1.5% of living patients presented with clinically identifiable GIT problems. Furthermore, a large majority of deceased patients (9 out of 12) possessed visceral anomalies. This suggests that the percentage of anomalies identified during clinical examination and history taking should be considered the minimum estimate. They suggest that clinically silent visceral anomalies, minor internal anomalies and other anatomic variations in syndromic craniosynostosis cases may be more prevalent than initial presentation suggests.

Our study also proposes that GI anomalies in the FGFR-associated craniosynostosis population may not be an infrequent finding. Over the past three and a half decades, six case reports have documented the finding of a GIT anomaly in FGFR-associated craniosynostosis patients. Some of these case reports illustrate the importance of early recognition of GIT malrotation as a possible comorbidity associated with FGFR-related craniosynostosis syndromes. Our study identified two PS patients and an AS patient with intestinal malrotation. One of the PS patients exhibited findings consistent with intestinal malrotation that was diagnosed during her second year of life. This patient had a jejunum that lay to the right of the midline, her cecum was abnormally located superomedially and the ascending colon was on a horizontal plane with the cecum directed medially. Another one of our identified PS was an infant who presented with PS and subsequently presented with a small bowel obstruction secondary to volvulus and a duodenal web. Fortunately, prompt diagnosis was able to avoid further morbidity in this case. Lastly, the AS patient with intestinal malrotation identified in this study was discovered later in life as a result of a history of recurrent vomiting and abdominal pain. These previous case reports and the three cases identified in this study highlight the importance of recognizing GIT malformation as a possible comorbidity to FGFR-related craniosynostosis syndromes.
3. Proposed mechanism

The anatomical development of the GIT is a complex process occurring in three distinct phases: i) an early phase of umbilical cord herniation, lasting from weeks 5-10 weeks of embryogenesis followed by ii) a stage of reduction of the midgut loop back into the abdomen occurring at weeks 10-11 and finally iii) a period of fixation from the 12th week of development lasting until after birth (Frazer & Robbins, 1915; Mall, 1898; Strouse, 2004). In the early embryo, the GIT is a straight tube supplied by the superior mesenteric artery (SMA). The midgut lengthens disproportionally to the embryo, forming a U-shaped loop that herniates into the base of the umbilical cord at approximately 6 weeks of gestation. Coincident with this growth, the small intestine rotates a total of 270 degrees in an anti-clockwise direction being completed by the 10th week when the intestine transitions back to the abdomen (Martin & Shaw-Smith, 2010). Malrotation of the intestine is a broad term used to collectively describe any failure of rotation that alters the normal developmental process of any part of the intestinal tract. It is not a single distinct entity, but rather a continuum of abnormalities occurring during development of the midgut. Martin and Shaw-Smith (2010) suggest that four etiologies may be attributed for intestinal malrotations. These include: i) abnormalities of left and right patterning, ii) abnormalities of the dorsal mesentery, iii) abnormalities of the bowel and iv) incorrect placement of the intestine or abdominal organs.

For our purposes, we can consider the proposed roles of FGF signaling during embryogenesis to suggest a link between FGFR-associated craniosynostosis and GIT malrotation. It has been shown that FGF signaling is crucial in many aspects of early embryogenesis, including the coordination of morphogenetic movements including dorsoventral, anterioposterior and left-right (L/R) axis determination (Dorey & Amaya, 2010). In the sea urchin both the fgfA ligand, and the fgfr2 receptor are necessary for the migration of the primary mesenchyme cells (Röttinger et al., 2008). Similarly in Drosophila, a mutation in the fgfr2 gene results in the failure of mesodermal cells to migrate away from the midline during gastrulation (Beiman et al., 1996; Gisselbrecht, et al., 1996). The L/R axis is the third axis to be established in the embryo and is responsible for the asymmetric placement of the thoracic and abdominal organs, including what is considered the normal positioning of the bowel (Dorey & Amaya, 2010).
Studies in mouse, chick, rabbit and zebrafish have demonstrated a direct role in FGF signaling in L/R axis determination (Albertson & Yelick, 2005; Boettger et al., 1999; Fischer et al., 2002; Meyers & Martin, 1999). In all vertebrates, the L/R axis appears to originate from a fluid flow in the node, a closed depression on the ventral surface of the embryo (Essner et al., 2002; Nonaka et al., 1998). This extraembryonic fluid undergoes a leftward flow that is set into motion by polarized monocilia and is thought to be responsible for normal situs (Tanaka et al., 2005). FGF signaling seems to play several important roles during this early step of L/R axis determination and may be responsible for the formation of cilia, cilia length (Neugebauer et al., 2009), and for the release of vesicular nodal parcels (VNPs) containing morphogens—Sonic hedgehog (SHH) and retinoic acid (RA)—into the node of the mouse (Tanaka et al., 2005). Any genetic mutation that could interfere with nodal fluid flow altering L/R patterning of the dorsal mesentery, the intestine and other abdominal contents may lead to intestinal malrotation.

Further support for this plausible proposed mechanism can be extrapolated from the Cohen and Kreiborg (1993) study of AS patients. These authors report many different visceral anomalies afflicting these patients including examples of L/R patterning problems such as pulmonary stenosis, dextocardia, mesocardia and dextrorotation in the cardiovascular system and partial biliary atresia with agenesis of the gallbladder in the GI system (Cohen & Kreiborg, 1993; Lindsay, Black, & Jr., 1975; Ofodile & Adeloye, 1982).

4. Study limitations

This study was retrospective in nature. Inherent limitations of retrospective study designs include the reliance on the accuracy of written record, which at times may be sparse or inaccessible. Although SickKids maintains a thorough record of all patients treated, while reviewing hospital records, inconsistencies in nomenclature and reporting errors were identified. We observed a probable documentation error in the charts of three CS patients who likely had a mutation recorded incorrectly. In these cases, the documented amino acid change did not match the genomic sequence; thus, we conclude that a typographical error must have occurred.

Furthermore, potential biases could exist in our sample population. It is possible that since SickKids is a leading paediatric hospital in North America, with a craniofacial program,
parents of children with craniosynostosis may tend to seek out a multidisciplinary center such as SickKids for all of their care. This could potentially increase the number of patients with intestinal malrotation seen at SickKids. Alternatively, and more likely, many patients travel great distances to seek care in the craniofacial program at SickKids, but when a GIT issue arises, these individuals may be more likely to see their local physician or hospital for relatively mild symptoms or because they may not associated the two problems of craniofacial and GIT anomalies together. Thus, it is quite possible that our sample may represent an underascertainment of the true incidence of rotation in this craniosynostosis population.
VI. FUTURE DIRECTIONS

The findings of this study suggest a correlation between FGFR-related craniosynostosis syndromes and GIT malformations. However, a direct cause and effect relationship cannot be ascertained. Further research will need to be conducted to confirm this relationship. While the findings of retrospective studies can be very useful in generating thought provoking data, they are only hypothesis generating, and thus are most useful to suggest future directions for research.

We proposed that an FGFR gene mutation is involved in the malformation of the gut solely based upon the evidence presented in current literature. Future directions for research could involve using a multi-center approach to look at a greater quantity of these FGFR-associated syndromic cases. Conducting a study to examine patients with craniosynostosis and a known FGFR mutation compared to patients with craniosynostosis but no FGFR mutation to discern any differences that are noted in the GIT may shed further light on the potential widespread impact an FGFR mutation can exert.
VII. CONCLUSION

Our study supports the theory that intestinal/bowel malrotations in an FGFR-associated craniosynostoses population are more likely to occur compared to the general population. According to the current management protocol, screening of GIT disorders is not a part of the investigative procedures in the care of CS patients. This study provides a sound scientific rationale for the possible implementation of changes, e.g., additional testing and screening procedures for GIT problems, in the clinical management of patients with FGFR-associated craniosynostosis.

The findings of our study support a plausible explanation for the discovery of GIT anomalies, specifically intestinal malrotation in craniosynostosis syndromes. The involvement of FGFR in the normal growth and development of the GIT provides a sound scientific rationale for the anomalies occurring in cases where mutations involving this gene occur. In addition, research in animal models, the few studies and case reports demonstrating GIT further supports the plausible correlation between FGFR-associated craniosynostosis and GIT malformations. This, we speculate that GIT anomalies are a direct result of mutations occurring in the FGFR altering the normal pathway of development and growth yielding malformations.
VIII. REFERENCES


Missense Changes, Insertions, and a Deletion Due to Alternative RNA Splicing. *American journal of human genetics, 58*, 491–498.


IX. Appendices

Appendix A – Hospital for Sick Children Research Ethics Board Approval

![Image of the document](image_url)

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<tr>
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<th>Department/Division</th>
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<tr>
<td>Dr. Sarah Bowdin</td>
<td></td>
<td>GENETICS</td>
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<td></td>
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<td>SickKids I.D. #: 2499</td>
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<td>Dr. Christine Hibberd</td>
<td></td>
<td>University of Toronto</td>
<td>Graduate Orthodontic Dept</td>
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<td>Dr. Christopher Forrest</td>
<td></td>
<td>SickKids</td>
<td>Plastic Surgery</td>
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These signatures confirm that each investigator has read the proposal and agrees to conduct this study in compliance with the Tri-Council Policy Statement, the Personal Health Information Protection Act (PHIPA), and any other applicable legislation and regulations, to adhere to the approved protocol, to apply to the Hospital For Sick Children (SickKids) Research Ethics Board (REB) for approval of amendments, report adverse events to the REB, submit annual reports and cooperate with any monitoring activities determined by the REB.

3. OTHER RESEARCH TEAM MEMBERS WHO ARE NOT CO-INVESTIGATORS (names of individuals who will be accessing personal health information e.g., health records/Electronic Patient Charts (EPC). Please print names (signatures not needed).

Name(s): Dr. Tompson and Dr. Gong

Position: Orthodontist, HSC; Orthodontist, UofT

SickKids Research Ethics Board

Short Application Form

May 2007
Appendix B - The Hospital for Sick Children Research Ethics Board reapproval letter
RESEARCH ETHICS BOARD

December 14, 2012

Dr. Sarah Bowdin
Clinical and Metabolic Genetics
The Hospital for Sick Children

Dear Dr. Bowdin:

Your study "Craniostenosis, Fibroblast Growth Factor Receptor Mutations and Gastrointestinal Malformations - A Possible Link."

REB File No.: 1000029124

On behalf of the REB, I am writing to confirm that the above noted study was re-approved by the REB for one year ending in December 2013. The REB approved continuing review at level 1A. As necessary, the Clinical Research Office will be contacting you to arrange follow-up.

Please note that, in accordance with the Personal Health Information Protection Act of Ontario, you are responsible for adhering to all conditions and restrictions imposed by the REB governing the use, security, disclosure, return and disposal of the research subjects' personal health information. You are also responsible for reporting immediately any privacy breaches to the REB Chair and to Janice Campbell, the Sick Kids privacy officer.

Yours truly,

Richard Sugarman
Chair, Research Ethics Board

Co-Investigator(s): Christopher Forrest, Christine Hibberd

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Toronto, Ontario
Canada M5G 1X8

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