Causes and Treatment of Velopharyngeal Insufficiency in 22q11.2 Deletion Syndrome

Oorzaken en behandeling van velopharyngeale insufficiëntie in het 22q11.2 deletie syndroom

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 30 juni 2011 des ochtends te 10.30 uur

door

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INTRODUCTION

Introduction

The 22q11.2 deletion syndrome (22q11.2DS) is a common microdeletion syndrome that occurs in between one in 4,000 to 6,000 live births [1,2]. It encompasses the phenotypes previously known as DiGeorge syndrome, velocardiofacial (VCF) syndrome, conotruncal anomaly face syndrome, and a subset of cases of the autosomal dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome (asymmetric crying facies). Patients with 22q11.2DS have a range of findings, including palatal abnormalities, conotruncal heart disease, characteristic facial features, immune deficiency, psychiatric problems, and learning difficulties.

Over 90% of 22q11.2DS patients have a *de novo* deletion. It has been demonstrated that the deletion endpoints cluster, and that there is a typically deleted region of 3Mb in 85% of patients [3]. Remarkable inter- and intra-familial clinical variability complicates genotype-phenotype correlations [4]. Possible mechanisms causing phenotypic variability may be modifier genes on the remaining allele of 22q11.2, elsewhere in the genome, epigenetic events, or chance.

Currently no accepted global protocol in the management of patients with 22q11.2 deletion syndrome exists though it is under development. As a rule, patients diagnosed with the 22q11.2 deletion usually undergo a series of evaluations, including cardiac, immunologic, otolaryngologic/audiologic, and developmental evaluations. When appropriate, speech and other developmental interventions are implemented.

Structural palatal abnormalities are found in approximately one third of patients with 22q11.2DS [5]. About 16% percent have a submucosal cleft, 11% have an overt cleft, and 5% have a bifid uvula. In addition, two thirds of 22q11.2DS patients exhibit symptoms of velopharyngeal insufficiency (VPI) such as hypernasal speech [5]. Consequently, pharyngeal surgery is often required to treat the excess nasal air escape. Surgeons aim to correct VPI by decreasing the size of the velopharyngeal gap. This can be done by lengthening the palate, mobilising a pharyngeal flap that spans the centre of the velopharyngeal gap but retains lateral ports, or rotating lateral

flaps to augment the pharyngeal sphincter. Previous studies have shown that the surgical outcome of pharyngoplasty in 22q11.2DS patients is less successful than that of non-syndromic patients [6,7,8,9,10].

In this study, possible reasons for poorer surgical outcome after pharyngoplasty in 22q11.2DS patients were explored. Therefore, the first research question in this thesis was: Are there anatomical or functional reasons in these patients that dictate relatively inferior results? In chapter one a number of factors that are likely to play a role in poorer speech outcome after pharyngoplasty in 22q11.2 DS patients are brought forward. The possible role of pharyngeal hypotonia is further investigated in chapter two of this thesis. Histopathological studies were done in pharyngeal muscle biopsies of patients with velopharyngeal insufficiency both with and without the 22q11.2 deletion. In chapter three, the results of a whole genome association study of candidate genes for palatal abnormalities in 22q11.2DS subjects are presented. It provides tentative evidence for the genetic mechanism of palatal dysfunction in the syndrome.

The second research question in this study was: How can we optimize the surgical treatment of velopharyngeal insufficiency in 22q11.2 DS patients? Chapters four and five describe the outcome of the surgical technique that is utilized in the Wilhelmina Children's Hospital in Utrecht, The Netherlands. In comparison, chapter six shows the outcome of the surgical techniques that are employed in the Children's Hospital of Philadelphia in the United States of America. Both medical centers have extensive experience in treating patients with 22q11.2DS. Evaluating techniques from both centers enabled us to study a larger group of patients and, more importantly, to directly compare the surgical techniques of two important treatment centers of 22q11.2 DS patients. The final chapter offers the conclusions of this study.

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CHAPTER 1

Possible mechanisms and gene involvement in speech problems in the 22q11.2 deletion syndrome

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Abstract

The 22q11.2 deletion syndrome represents a contiguous gene syndrome with a highly variable phenotype. To date, over 180 clinical features have been described. Studies have been done in order to identify the responsible genes. Several candidate genes such as *Tbx1* and COMT seem to be important in the development of the phenotype. One of the prevalent and serious problems encountered by patients with the 22q11.2 deletion is difficulty with speech. This may be due to a number of factors such as adenoid hypoplasia, muscle hypotonia, platybasia, upper airway asymmetry, and neuroanatomical abnormalities. The complex interaction of these factors leads to less favourable results after surgery to correct velopharyngeal insufficiency. This article offers a theoretical overview and proposes future research to investigate which factors are indeed responsible for the speech problems encountered by patients with the 22q11.2 deletion and identify responsible genes.

Introduction

The 22q11.2 deletion syndrome (22q11.2 DS) is also known as velo-cardio-facial syndrome (VCFS), DiGeorge syndrome, conotruncal anomaly face syndrome and Shprintzen syndrome. It was recognised in the early nineties that most cases of DiGeorge syndrome and virtually all cases of VCFS resulted from a common deletion of the long arm of chromosome 22. A minority of the 22q11.2 deletions is cytogenetically visible in conventional karyotyping. In other cases, the deletion can be detected by fluorescence in situ hybridization (FISH) or by multiplex ligation-dependent probe amplification (MLPA). The mode of inheritance of the 22q11.2 deletion is autosomal dominant but in the majority of patients, approximately 85%, the deletion occurs *de novo*. The estimated prevalence of 22q11.2 DS varies between one in 2000 and one in 6000 new-borns [1,2, 3] . No significant gender or ethnic differences have been found [4]. An important confounding factor in studies of the prevalence of the 22q11.2 deletion is the possibility for mild or late onset of symptoms. Therefore the actual prevalence is possibly underestimated. One of the characteristic features of 22q11.2 DS is the presence of speech difficulties. This article describes these difficulties and their management in patients with the deletion. In addition, the possible genetic basis for these speech pathologies is discussed.

Clinical features

To date, over 180 clinical features have been associated with the deletion [2, 3] (see Table 1). The features show great inter- and intrafamilial variability. This variability is independent of the size of the deletion [5]. Physical manifestations have been described which involve every organ system. The first presenting symptom is usually a congenital heart defect. Characteristic facial features are small ears, flat cheeks, a bulbous nose, hypertelorism, and almond shaped eyes (see Figure 1). An estimated 69 percent of patients with the 22q11.2 deletion have palatal features that include overt, submucous, or occult submucous clefts of the secondary palate, and velopharyngeal insufficiency [6]. About 5 percent of all children with cleft palate has the deletion. This makes 22q11.2 DS the most common syndromic cause of cleft palate. However, genetic testing of the locus in all patients with cleft palate alone is not recommended [7]. Only when other symptoms besides palatal abnormalities are present is genetic testing for the deletion indicated.

Craniofacial/Oral findings	**Eye findings**	**Problems in infancy**
Cleft palate	Narrow palpebral fissures	Feeding difficulty
Platybasia	Mild orbital hypertelorism	Failure to thrive
Retrognathia	**Ear/hearing findings**	Nasal vomiting
Small teeth	Overfolded helix	**Cognitive/learning**
Asymmetric crying face	Attached lobules	Learning disabilities
Hypotonic facies	Protuberant, cup-shaped ears	Borderline normal intellect
Downturned commissures	Frequent otitis media	Mild retardation
Pharygeal/laryngeal findings	**Nasal findings**	**Psychiatric/psychologic**
Absent or small adenoids	Prominent nasal bridge	Bipolar disorder
Laryngeal web	Bulbous nasal tip	Psychosis
Large pharyngeal airway	Narrow nostrils	Depression, hypomania
Laryngomalacie	**Extremities**	Schizoaffective disorder
Pharyngeal hypotonia	Small hands and feet	Schizophrenia
Asymmetric pharyngeal movement	Triphalangeal thumbs	Attention deficit hyperactivity disorder
Thin pharyngeal muscle	Polydactyly	**Neurologic findings**
Speech/language	Soft tissue syndactyly	Generalized hypotonia
Severe hypernasality	Hyperextensible joints	Seizures, strokes
Articulation impairment	**Cardiologic findings**	Mild developmental delay
Language impairment	Ventricular septum defect	**Endocrinological findings**
Velopharyngeal insufficiency	Atrial septum defect	Hypocalcemia
	Tetralogy of Fallot	Hypo(para)thyroidism
		A-/hypoplastic thymus

Table 1. Findings associated with the 22q11 deletion syndrome.

Most children show both developmental delay and learning difficulties and often need special education. Their learning disabilities are both verbal (language, speech, articulation, reading, comprehension) and non-verbal (motor skills, math, visuo-spatial organisation). The majority has mild or moderate retardation and average full scale IQ is approximately 75. Also, behavioural and psychiatric disorders are common in children with 22q11.2 DS [8]. Behavioural problems include emotional instability, social withdrawal, attention-deficit/hyperactivity disorder, anxiety disorder, and depression. Psychiatric disorders, primarily autistic disorder, schizophrenia, paranoid delusions, and bipolar disorder develop in 25 to 50 percent of cases. Schizoaffective disorder, schizotypal personality, obsessive-compulsive disorder, and anxiety disorder are also common [9]. In addition, children with 22q11.2 DS have been found to have significant overall reductions in brain volume, compared to the development of normal children [10].

New symptoms and characteristics associated with 22q11.2 DS are described regularly. Recently it has been suggested that there is a higher risk of malignancy in 22q11.2 DS patients [11]. Another research group reported on the possible higher prevalence of tracheobronchial anomalies when compared to healthy controls [12]. This illustrates how important it is to remember that many different abnormalities in all organ systems can be attributed to the 22q11.2 deletion.

Management

Currently no accepted global protocol in the management of 22q11.2 deletion syndrome exists. After the international 22q11.2 deletion conference in Marseilles, France in 2006 this is under development. In the Netherlands a national guideline for the management of 22q11.2 DS has been in use for several years [13]. As a rule, patients diagnosed with the 22q11.2 deletion usually undergo a series of evaluations, including cardiac, palatal, immunologic, otolaryngologic/audiologic, and developmental evaluations. When appropriate, speech and other developmental interventions are implemented. Pharyngeal surgery is frequently required in patients with palatal abnormalities to treat the nasal air escape that causes hypernasality. As indicated by the specific clinical presentation neurologic, endocrinologic, or other evaluations may also be considered. In this multitude of assessments in various fields of medicine it should be clear for the patients and their families where they can go for questions and information regarding treatment programs. This role can be carried out by a single specialist, i.e. a

paediatrician or paediatric plastic surgeon, or by a team specialised in the care of children with the deletion. Ideally, children with 22q11.2 DS and their parents are able to see all the specialists involved in their treatment in either one session or one day. Regular deliberation between the various professionals should be facilitated. In most countries, support groups exist for patients and their families that aid in organized care and education (for additional information see [14]).

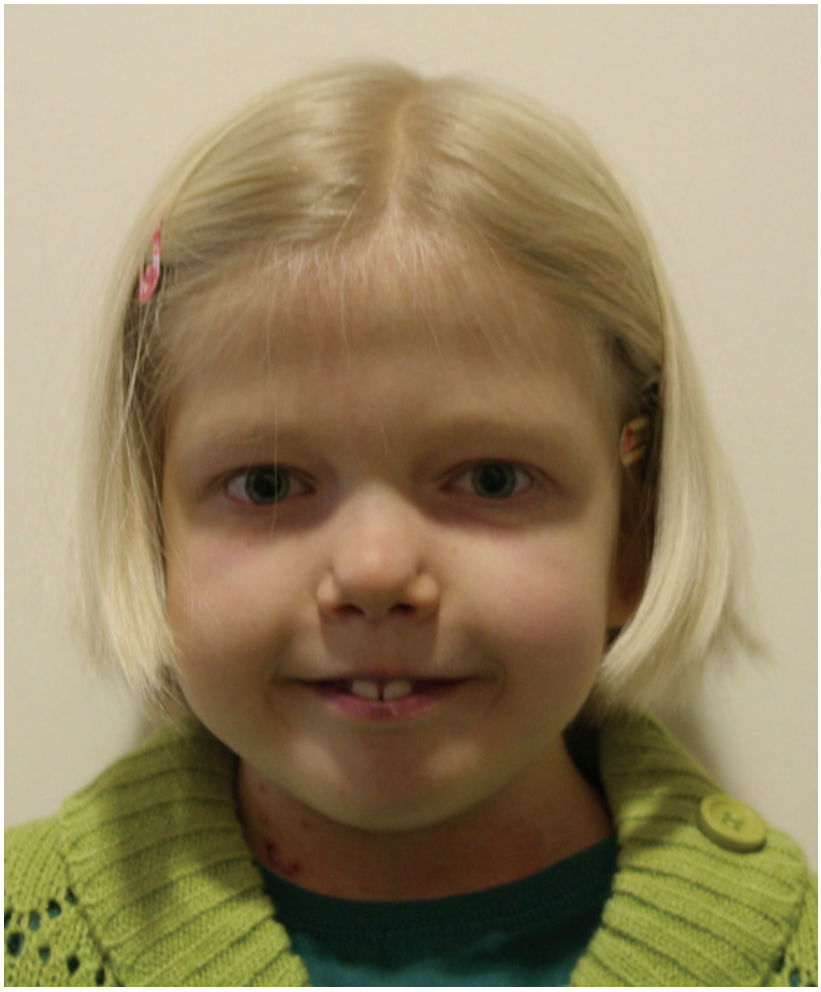

Figure 1. Nine-year-old female patient with typical facial dysmorphic features associated with the 22q11.2 deletion.

Speech difficulties

One of the characteristic features of 22q11.2 DS is the presence of speech difficulties. Several studies have shown that children with the deletion show delayed language onset [15, 16, 17]. In addition, a number of other communication disorders has been described. These include articulation, language, resonance, and voice problems. Palatal abnormalities may cause hypernasal speech and nasal air emission. Secondary to palatal anomalies and velopharyngeal insufficiency (VPI) compensatory articulation errors occur. The articulatory errors observed in children with 22q11.2 DS seem not only related to a delay in speech sound acquisition as they are uncharacteristic of normal speech development, although it is not yet clear if they are syndrome-specific [18]. Aggravating these speech difficulties are language problems due to cognitive deficits, delayed motor development, and frequent hearing problems [16]. The incidence of middle ear disease varies among different authors: probably as a result of an ascertainment bias Shprintzen et al. [19] reported an incidence of 77%, while Finkelstein et al. [20] mentioned that only 22% of his patients had middle ear problems. Chronic serous otitis media results in hearing loss of the conductive type. A minority of the patients with 22q11.2 DS has a small degree of sensorineural hearing loss [19]. Prolonged bilateral hearing loss of 25 dB or more may obviously influence speech and language development. However, specific and systematic knowledge regarding the ear disease in this syndrome is still scarce.

Pharyngoplasty

The 22q11.2 deletion is the most common syndrome associated with clefting. Patients with an overt or submucous cleft palate should preferably have their palate repaired in the first year of life, including repair of the levator sling. Due to the anatomical disparities found in 22q11.2 deletion syndrome VPI may persist after closure of the palate. In these cases further surgical correction is indicated. VPI may also exist without clear anatomical abnormalities of the palate (due to muscle hypotonia etc) [21]. Most surgeons opt for velopharyngeal narrowing procedures using either a posterior pharyngeal flap or a sphincter pharyngoplasty in the treatment of VPI [22, 23, 24, 25]. In our hospital a palatal lengthening procedure is used. So far, there is no general consensus as to which surgical technique is preferable. Preoperative analysis of the individual anatomical defect using videofluoroscopy and nasendoscopy is mandatory. This will help assess the velar function, the depth of the pharynx, the shape of the anterior wall, and the size of the

velopharyngeal gap. Both before and after surgery speech and language pathologists familiar with 22q11.2 DS play an important role in the development of satisfactory speech patterns.

Patients with the 22q11.2 deletion often have less satisfactory outcomes after VPI-correcting surgery than their non-syndromic counterparts [22, 23, 25]. Objective speech data often demonstrate greater preoperative velopharyngeal insufficiency than patients without the deletion and the need for revision after surgery is higher. When compared to a group of children undergoing pharyngoplasty for VPI without the deletion, the need for revision after primary surgery was twice as high in the group of children with the deletion (11% versus 22%) [23]. The exact reasons for these poorer results remain unclear. To date, a number of possible explanations have been proposed on the basis of the anatomical and functional differences observed in 22q11.2 DS which will be discussed in the following sections.

Adenoid Hypoplasia

It has been observed that the adenoid is the primary site of contact for the velum during speech in children below the age of 5 or 6 years [26]. The increased VPI associated with the 22q11.2 deletion may, at least in part, be due to hyoplasia of the adenoids found in many of these patients. In 2000, Havkin and her colleagues found that smaller adenoid size did indeed correlate with abnormal articulation patterns [26]. Therefore, imaging studies such as nasendoscopy and multi-view videofluoroscopy are necessary to determine the contribution of the adenoids to velopharyngeal function before pharyngoplasty.

Muscle Hypotonia

In 1995, patients with 22q11.2 DS with hypodynamic or paretic velopharyngeal mechanisms were described by Witt et al [27]. Consequently, the importance of muscle hypotonia as a cause of VPI in 22q11.2 deletion syndrome was shown in a study by Zim et al. in 2003 [28]. The thickness of the superior pharyngeal constrictor muscle they found to be significantly less in patients than in control subjects. Patients with the 22q11.2 deletion also had fewer type II muscle fibres in pharyngeal muscle specimens compared to controls. As the superior pharyngeal constrictor muscle normally has a large proportion of type II fibres, the relative lack of these fibres in 22q11.2 deletion patients may contribute to their problems with velopharyngeal

insufficiency and speech. Due to the hypotonic nature of the lateral pharyngeal walls in patients with 22q11.2 DS sphincter pharyngoplasty might be the preferred treatment above posterior pharyngeal flaps [23].

Platybasia

Platybasia is defined as developmental deformity of the occipital bone and upper end of the cervical spine, in which the latter appears to push against the floor of the occipital bone causing it to bulge upwards. As a result, the basal angle of the skull is increased, causing the skull base to be literally flatter. This enlarges the velopharyngeal gap that needs to be closed during surgery making it more difficult with a higher risk of complications and disappointing outcome. A study was published in 2004 in which a cross-sectional cohort of children with the 22q11.2 deletion were evaluated on variations of the occiput and cervical spine [29]. Platybasia was found in fifty-two of fifty-seven patients (91%). The clinical importance of this finding is unclear, as platybasia is not considered to be an abnormality that warrants concern. However, it does alter the anatomy of the nasopharynx and oropharynx and could thereby add to the more severe velopharyngeal insufficiency in 22q11.2 deletion patients.

Upper Airway Asymmetry

Recently, it has been reported that there may be a high prevalence of upper airway asymmetry in patients with 22q11.2 DS [30]. An asymmetry was found in 69% of patients, compared to 20% in controls. Asymmetry in palate elevation was found to lead to incomplete closure of the velopharynx on the side with less elevation. Combined with the previous findings of pharyngeal muscle hypotonia these data allude to an abnormality related to the muscle development in this area. Moreover, although not significant, a relatively high rate of abnormal vocal cord size and motion (38%) was also found. It should be taken into account that this could also be a factor in the speech problems. Hence, all patients with the 22q11.2 deletion and speech problems need an extensive evaluation of both the anatomy and function of the upper airway prior to treatment with videofluoroscopy and/or nasendoscopy.

Neuroanatomical Anomalies

Apart form aberrations in the anatomical structures involved in the execution of speech, anomalies in stimulation through the central nervous system could aggravate or elicit the problems with speech in 22q11.2 DS. A range of variations in grey matter and white matter

volume and connectivity have been associated with the deletion [31]. One study with 22q11.2 DS patients did show significant bilateral thinning of the cortex in a portion of the inferior frontal gyrus, an important area for language development [32]. Hypotonia and motor delays may be associated with the cerebellar abnormalities that have also been described in the syndrome (small cerebellar vermises and/or small posterior fossa) [33]. A correlation might also be made between the enlargement of the sylvian fissure and the marked speech delays that typify VCF. Children without the syndrome but with similar neuroanatomic abnormalities known as 'congenital bilateral perisylvian syndrome' show oromotor dysfunction, including nasal speech, dysarthria, dysphagia and drooling, which all are common in 22q11.2 DS [34]. This may suggest that irregularities in the brain do play a part in the development of speech problems seen with the deletion. However, the relation of the behavioral phenotype of 22q11.2 DS to its neuroanatomic features remains speculative [34].

Additional factors complicating surgery

Not only the aforementioned anatomical disparities can influence the outcome of children with the 22q11.2 deletion after surgery, but a number of other factors may also complicate surgical management. A host of comorbid conditions may be present in patients with deletion, such as cardiac anomalies and immunodeficiencies, making both the pre- and postoperative procedures more complex.

In addition, the carotid arteries frequently follow an anomalous course that can make pharyngeal surgical procedures more difficult. One in five patients with 22q11.2 DS has a medial displacement of one or both internal carotid arteries. In a subset of these cases the artery is located in the donor site for the pharyngeal flap where the vessel is at risk for injury during surgery. Nevertheless, it has been shown that surgery can be carried out safely [35], which is consistent with our own experience. In most patients with a displaced artery it will lateralize when the neck is extended during the procedure. However, some surgeons still recommend magnetic resonance imaging to be carried out prior to surgery [36].

Timing of surgery

Although there is debate about the optimal age to carry out pharyngoplasty [22] it is generally believed that a later age at time of surgery may lead to a higher revision rate and poorer outcome. Hence, the fact that children with 22q11.2 DS without directly noticeable anomalies

are usually diagnosed at a later age may also be of influence on the problems encountered after surgery.

Speech Therapy

In 22q11.2 DS the psycho-educational profile related to speech-language acquisition and learning difficulties may limit the efficacy of speech therapy. Despite their delay in speech - language development, school-aged children with the deletion typically show higher Verbal IQ than Performance IQ scores [37, 38]. This is consistent with a nonverbal learning disability that is rare in the general population [34]. Reading and Spelling achievement scores are higher than Mathematics scores (Wechsler Intelligence Scales for Children) in almost 90 % of patients, with a mean difference of almost 10 points [37]. Full-scale IQ is typically in the low-normal to borderline range among school-aged children with 22q11.2 DS, with Verbal IQ averaging close to 80, Performance IQ close to 70, reading and spelling achievement scores in the high 80s, and mathematics scores in the low 80s [34]. These findings provide to some extent a psychoeducational profile of 22q11.2 DS, as has been described for other genetic syndromes such as fragile X and Prader-Willi. Dykens [39] identified these profiles as one facet of the behavioral phenotype, which can be of clinical use in the diagnostic process and in patient management.

Genetic Basis

The majority of patients who are clinically suspected of 22q11.2 DS and subsequently undergo genetic testing have a 3.0 megabase deletion. Less than 10% of patients has a smaller 1.5 megabase deletion and only few cases have unique smaller deletions [5].The most commonly deleted region of 22q11.2 is known as the DiGeorge Chromosomal Region (DGCR) and comprises approximately 2.0 megabases (see Figure 2). It can be assumed that haploinsufficiency of one or more genes in this region is responsible for the aetiology of VCFS and DiGeorge syndrome. In recent years a number of candidate genes has been identified. However, no clear relationship between genotype and phenotype has been established yet.

Tbx1

In order to identify candidate genes responsible for the clinical features associated with 22q11.2DS, studies have been carried out using mice which carry mutations on chromosome 16 (MMU16) corresponding to the human 22q11.2 region [40, 41, 42]. From these studies, *Tbx1*, a member of the T-box family of transcription factors, was found to play a role in the development of the 22q11.2 deletion phenotype. *Tbx1* is expressed during early stages of embryogenesis in the pharyngeal arches. Mice carrying a mutation in the *Tbx1* gene showed a heterozygous phenotype with abnormal aortic arch arteries. In comparison, no vascular abnormalities were observed in the wild type embryos [41]. Additionally, mice with a homozygous deletion in *Tbx1* displayed a wide spectrum of phenotypic characteristics commonly associated with the 22q11.2 deletion in humans, though more severely. These homozygous mutant mice all died at birth; they were oedematous, indicating cardiovascular insufficiency, and appeared smaller than the heterozygotes. Besides that, all the homozygous mutants had a cleft palate as the bony elements of the palatine and maxillary shelves were never fused [42]. It is striking that this range of abnormalities is not observed in heterozygous *Tbx1* mutant mice since VCFS in humans is a haploinsufficiency syndrome. In humans heterozygous deficiency is sufficient to cause the clinical features. This could be explained by a species dependent difference in sensitivity to *Tbx1*. In that case, humans must be increasingly sensitive to low levels of the gene product when compared to mice.

Further insight in the role of *Tbx1* in the clinical features of 22q11.2 DS was provided by a study in which subjects with phenotypic characteristics of the 22q11.2 deletion syndrome but without the actual deletion were studied. In this group three mutations in *Tbx1* were found which were not found in 555 healthy controls [43]. Evidence exists that mutations resulting in both gain-of-function as well as loss-of-function of *Tbx1* cause some of the phenotypic characteristics of 22q11.2 DS [44]. This suggests that *Tbx1* does indeed play an important role in the 22q11.2 deletion syndrome, as mutations in the single gene can cause a clinically similar phenotype. Interestingly, though these subjects with a mutation in *Tbx1* did display abnormalities of the heart, palate, thymus, and parathyroid glands, facial anomalies and a pharyngeal phenotype typical for 22q11.2 DS they did not have learning disabilities, which are almost always associated with 22q11.2 deletion. This indicates that abnormalities in *Tbx1* may lie behind a substantial part of the features of the clinical phenotype of 22q11.2 DS, but that there must also be other influential etiological genes.

Other Genes

In the past ten years, another number of possible candidate genes for the phenotype of the 22q11.2 deletion syndrome have been identified. One of the genes in the deleted region is COMT. It codes for catechol-O-methyl-transferase and plays a role in the metabolic degradation of synaptic dopamine and norepinephrine. Aberrant COMT levels as seen in 22q11.2 lead to abnormal levels of certain synaptic neurotransmitters and may thus partially explain the cognitive and behavioural problems associated with the deletion syndrome [45]. Other genes that potentially contribute to the 22q11.2 phenotype are *HIRA,* which is expressed in the neural crest, mesenchyme of the head and branchial arches, RanBP1 which is expressed in frontonasal processes, branchial arches, aortic arches, and limb buds, and *Gscl,* which is expressed in the thymus [42, 46]. In addition, a single significant association was recently found between a variant of MTR-gene and dysfunction of the velopharyngeal structures [47]. MTR encodes methionine synthase, which catalyses the remethylation of homocysteine to methionine. It appears that, though the roles of these genes may not be as substantial as *Tbx1*, they do affect developmental mechanisms that are disturbed in VCFS and DiGeorge syndrome. Either involved in independent pathways or in cascades with other disease genes, they generate the host of symptoms that accompany 22q11.2 DS. This genetic syndrome should thus be

considered a continuum where the complexity of gene-gene interactions may explain the clinical inter- and intrafamilial variability.

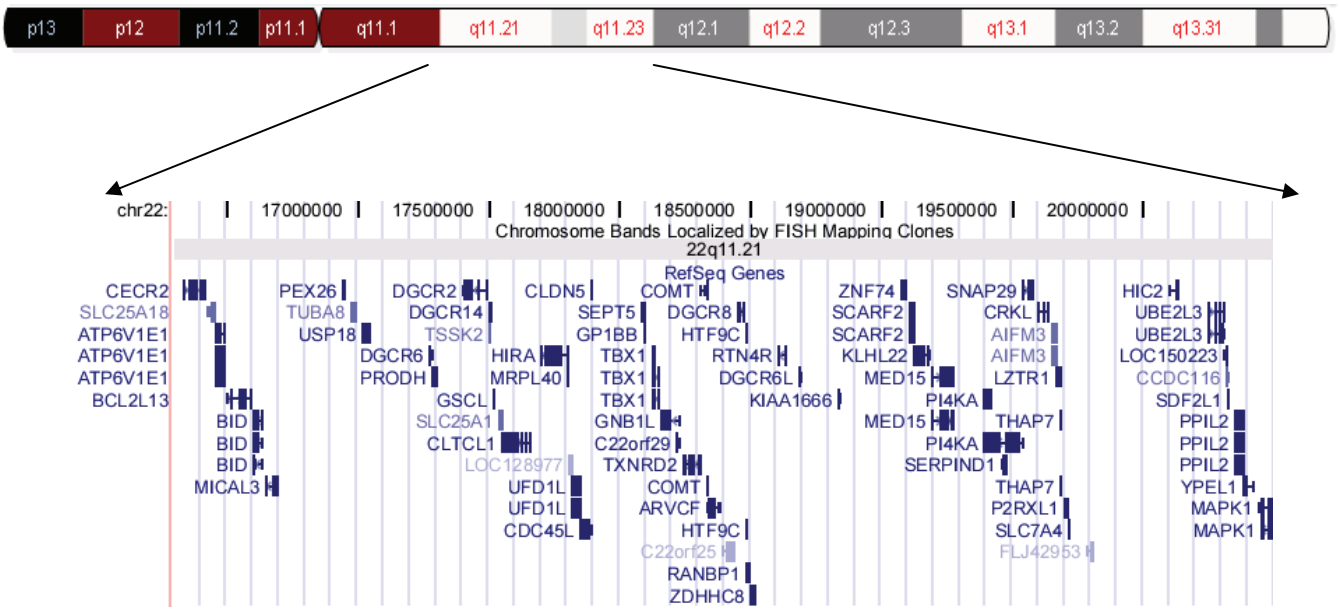

Figure 2. Chromosome 22q11.21: 16,300,001-20,500,000 (bp). March 2006 human reference sequence (NCBI Build 36.1) as produced by the International Human Genome Sequencing Consortium (UCSC Genome Browser accessed on September 24, 2007).

Conclusion

The 22q11.2DS has a wide range of clinical features expressed in varying severity. A number of candidate genes has been identified that may be implicated in the development of symptoms. Patients, their families, and health care professionals face a multitude of challenges with different clinical features arising at different times during life. The treatment and management of patients with the 22q11.2 deletion is a challenge, even for a multidisciplinary team.

The speech problems found in patients with 22q11.2 DS may be due to a number of anatomical and functional disparities such as cleft palate, adenoid hypoplasia, muscle hypotonia, platybasia, and upper airway asymmetry. Recently, a study was done using three-dimensional MRI imaging to assess the pharyngeal anatomy of 5 patients with 22q11.2 DS and indeed a flatter cranial base angle and wider velopharynx were found [48]. It could be hypothesised that these occurrences are (in)directly caused by a defect of one or more genes in the deleted region. The *Tbx1*gene appears to be a likely candidate as it is expressed in the pharyngeal arches. In future research, the authors plan to investigate whether *Tbx1* and other genes in the deleted region are associated with clefting of the secondary palate, surgical outcome and speech problems. Possible correlations between genotype and phenotype will be examined.

The various phenotypical presentations of palatal and speech problems in 22q11.2 DS are not yet exactly described. To date it remains unclear whether findings such as platybasia, adenoid hypoplasia, and upper airway asymmetry are found in *all* patients with the deletion or only in those with speech difficulties. The different phenotypes in 22q11.2 DS can be distinguished using comprehensive speech assessments and imaging studies detailing how the velopharynx functions when speech is produced. Only when these phenotypes are well documented can associations be made with the genetic profiles found in 22q11.2 DS.

A potential contributing factor to the communication problems in children with 22q11.2 DS are anomalies in neuroanatomy and function. A possible candidate gene for these abnormalities is COMT as it has already been associated with some of the cognitive features of the 22q11.2 deletion syndrome. Future studies should be designed to elucidate the association between the activity of COMT and other genes in the deleted region, possible related aberrant neuroanatomy and neurofunction, and the presence of speech problems. Currently, the authors plan to investigate the neuronal brain activity during speech production in subjects with 22q11.2 DS after pharyngoplasty and compare this to subjects without the deletion. Discrepancies in brain activity could point to underlying differences in anatomy and function in 22q11.2 DS and provide more insight into the less satisfactory results after pharyngoplasty. In the search for optimum treatment of children with the deletion it will be important to know whether the higher revision rates of VPI surgery and poorer speech and language skills found in 22q11.2 DS are related to neuroanatomical variations.

In conclusion, more research needs to be done documenting and investigating the mechanisms behind the speech problems in 22q11.2 DS. Our goal is to improve the chances of a favourable outcome for the individual 22q11.2 patient with speech problems. So far we have been largely unable to determine pre-operative characteristics which may help us to predict the outcome and to tailor VPI surgery individually for these patients. Being able to do so in the future would be a major step forwards. To achieve this, we recommend that, in order to evaluate the results of speech correcting surgery and therapy, all children with the 22q11.2 deletion need to undergo comparable pre- and postoperative assessments using imaging techniques and thorough speech evaluation in combination with genetic mapping of the deletion. This will facilitate meta-analysis of study outcomes and can help identify the optimal surgical technique(s), timing of surgery, and accompanying speech and language therapy. From this information a widely accepted standard protocol for the treatment of VPI and speech problems in 22q11.2 DS can be developed. We may find that the preferred treatment programme depends on variations in genetic profile or velopharyngeal phenotype, but it is possible that the level of experience of the surgeon will remain the dominant factor for success. In either case the knowledge gathered from unified pre- and postoperative evaluations will help in the management of this complex and variable syndrome.

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CHAPTER 2

Histology of the pharyngeal constrictor muscle in 22q11.2 deletion syndrome and non-syndromic children with velopharyngeal insufficiency

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Abstract

Plastic surgeons aim to correct velopharyngeal insufficiency manifest by hypernasal speech with a velopharyngoplasty. The functional outcome has been reported to be worse in patients with 22q11.2 deletion syndrome than in patients without the syndrome. A possible explanation is the hypotonia that is often present as part of the syndrome. To confirm a myogenic component of the etiology of velopharyngeal insufficiency in children with 22q11.2 deletion syndrome, specimens of the pharyngeal constrictor muscle were taken from children with and without the syndrome. Histologic properties were compared between the groups. Specimens from the two groups did not differ regarding the presence of increased perimysial or endomysial space, fiber grouping by size or type, internalized nuclei, the percentage type I fibers, or the diameters of type I and type II fibers. In conclusion, a myogenic component of the etiology of velopharyngeal insufficiency in children with 22q11.2 deletion syndrome could not be confirmed.

Introduction

The 22q11.2 deletion syndrome (22q11.2DS) is the most common human microdeletion syndrome [1] with an estimated frequency around 1 in 4000 [2] but possibly as high as 1 in 2000 surviving newborns[3]. It encompasses the phenotypes previously known as DiGeorge syndrome, velocardiofacial syndrome, conotruncal anomaly face syndrome, many cases of the autosomal dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome (asymmetric crying facies). Over 180 clinical features including every organ system have been associated with the deletion[4].

One of the presenting features of 22q11DS is velopharyngeal insufficiency (VPI). Velopharyngeal insufficiency is the failure of the soft palate to reach the posterior pharyngeal wall to close the opening between the oral and nasal cavities, resulting in hypernasal speech. Incomplete velopharyngeal closure is most frequently related to structural abnormalities such as cleft palate or submucous cleft, but may also be the corollary of neuromuscular impairment[5]. Both seem to be factors in the etiology of VPI in patients with 22q11.2 deletion syndrome where palatal defects, adenoid hypoplasia, and platybasia enlarge the pharyngeal gap [6], and the hypodynamic pharynx as viewed by nasendoscopy has been described as a "black hole"[7].

Surgical repair of palatal clefts does not sufficiently correct VPI in 10- 31.8% of all patients with VPI not restricted to those with 22q11DS [8,9,10,11,12], possibly due to stiffness or shrinkage of the velum due to scarring[5]. Secondary velopharyngoplasty to correct the VPI may then follow. The functional outcome has been reported to be worse in patients with 22q11DS than in patients without the syndrome [13,14,15,16,17,18]. A possible explanation is the hypotonia that is often present as part of the syndrome and which cannot be corrected by surgery.

Velopharyngeal closure is achieved by the concert action of multiple muscles, including palatal lift by the levator veli palatini and circular pharyngeal closure by the pharyngeal constrictor muscle (PCM)[19,20]. A previous study of the PCM shows that patients with 22q11DS have

proportionally more type I fibers and the diameter of these fibers is smaller than those in people without the syndrome[21]. In the study by Zim et al, muscle biopsies from children were compared with specimens from elderly cadavers.

Muscle fiber hypoplasia or atrophy with subsequent pharynx hypotonia may be primarily myogenic or neurogenic. Muscular and neurologic problems have been associated with 22q11DS both clinically and genetically. Specific myopathies are rare [22,23,24], but neurologic disorders including delayed motor and mental development [25,26,27] and dysfunction of cranial nerves III, VII, VIII, IX, X, and XII [28] affect at least 33% of patients [29,30]. General hypotonia, which affects 23-76% of patients with 22q11DS [29,31,32], was found to be universally prevalent among children with 22q11DS and VPI [33].

About 40 genes [3], including TBX1, are located in the 3.0 megabase region deleted in 22q11DS [1], affecting countless downstream signaling pathways. The central roles of the TBX1 and CRKL genes in the anomalous developmental of pharyngeal structures in 22q11DS have recently been reviewed [34]. The murine Tbx1-/- model for 22q11DS has hypoplastic branchiomeric muscles [35,36], but the sporadic muscles that develop have a normal distribution of muscle fibers types [37]. In patients with 22q11DS, decreased PCM muscle thickness on MRI [21] suggests hypoplasia. The temporal Tbx1 gradient follows the cranial-caudal development of pharyngeal structures [36], causing structures that are derived from more cranially located pharyngeal arches, such as the levator palatini muscles, to be less affected by the mutation than structures derived from more caudally located pharyngeal arches, such as the PCM muscle [38,39]. Although Tbx1 is not expressed in primary neural crest cells [40], Tbx1 mutants have aberrant structures derived from neural crest cells including cranial nerves [38] since defective Tbx1 expression in the pharyngeal endoderm affects the downstream expression Fgf8 and Fgf10 which are necessary for neural crest cell migration [38,41,42]. As suggested by studies on the deleted TBX1 gene [35,37,38], primary aberrant myogenesis leads to aberrant neurogenesis.

In summary, the poorer functional outcome after velopharyngoplasty in patients with 22q11DS may be attributed to pharyngeal hypotonia. Anomalous myogenesis and neurogenesis which may underlie the hypotonia have been reported in a murine model for 22q11DS. In this study we aimed to confirm a myogenic component of the etiology of VPI in children with 22q11DS by analyzing the histology of the PCM muscle. Our clinical experience is that the PCM seems thicker in children with 22q11DS. We expect to find fiber hypertrophy as a corollary of the muscle hypoplasia [35,36] necessitating the few fibers present to take on a heavier workload.

Methods

Patients

The University Medical Centre in Utrecht is the Dutch national centre for children with 22q11DS. Children undergoing velopharyngoplasty for VPI with and without the 22q11DS were included in the study. Children with contra-indications for velopharyngoplasty (including bleeding disorders or extensive comorbidity such as cardiac problems) and known neurological disorders were excluded. This study was approved by the institutional medical ethics review board and the patients' parents gave written informed consent to participate.

Sample size calculation

Using the results of the only previous study on PCM histology in 22q11DS [21] which found a difference of mean diameter of type I fibers of 5.0 μm between patients with and without 22q11DS, with a standard deviation of 2.0 μm, an alpha of 0.05, and a power of 0.80 in the two-tailed two-sample t-test sample size formula yields a sample size of 4 subjects in each group. This number was arbitrarily doubled as the difference between two groups of children is likely smaller than the difference between children and adults in the previous study.

Muscle specimens

During velopharyngoplasty, a cranially attached pharyngeal flap (measuring around 10 x 40-50 mm) is mobilized from the dorsal pharyngeal wall and attached to the velum. This flap is comprised of part of the PCM muscle and the overlying mucosa. Muscle at the caudal end of the flap is trimmed (measuring around 10 x 3 mm) and delivered fresh to the pathologist in a damp gauze for histological evaluation.

Outcome parameters

Histological evaluation of the muscle specimens included qualitative analysis and quantitative measurements. The analysts were blinded for age, gender and presence of the syndrome. The specimens were qualitatively evaluated for the presence of increased perimysial and endomysial space, muscle fiber grouping by size or type, and presence of internalized nuclei. After staining with ATPase at pH 4.3, representative areas from each specimen were photographed. For quantitative analysis, muscle fibers were counted and the percentage of type I muscle fiber was calculated per patient. The diameters of up to 100 fibers of each type were measured for each patient. For each muscle fiber type, the mean fiber diameter and variance ((SD x 1000)/mean diameter) were calculated per group (males, females, and children with and without 22q11DS).

Statistical analysis

The genders of children with and without 22q11DS were compared using the Chi-square test. Age at surgery of males and females and children with and without 22q11DS were compared using the Independent samples t-test. The presence of increased perimysial and endomysial space, muscle fiber grouping by size and type, and internalized nuclei was compared between the two groups using Fisher's exact test. The relationship between age at surgery and fiber diameters was examined using the Spearman correlation. The independent samples t-test was used to compare the mean percentage of type I fibers and muscle fiber diameters between males and females and between children with and without 22q11DS.

Results

Patients

Muscle specimens were available for 16 children, eight with 22q11DS and eight without 22q11DS. The groups did not differ regarding gender (5/8=63% and 4/8=50% female, respectively, p=0.63) or age at surgery (6.5 and 7.0 years, respectively, p=0.68) (Figure 1). Males and females did not differ regarding age at surgery (7.4 and 6.2 years, respectively, p=0.39).

Qualitative analysis

No structural differences were seen between histological specimens from children with and without 22q11DS (Table 1, Figure 2). Increased perimysial and endomysial space was seen equally in both groups. No grouping by muscle fiber type was seen in any patient. One non-syndromic patient had localized grouping of smaller fibers, but these were round fibers without nuclear clumping which do not suggest neurogenic atrophy or other signs of fiber degeneration and regeneration. One patient with 22q11DS had an increased percentage of internalized nuclei.

Parameter	22q11DS (n=8)	No 22q11DS (n=8)	p
Increased perimysial space, No. (%)	5 (63)	5 (63)	1
Increased endomysial space, No. (%)	4 (50)	6 (75)	0.61
Grouping by size, No. (%)	0 (0)	1 (13)	1
Grouping by fiber type, No. (%)	0 (0)	0 (0)	1
Internalized nuclei, No. (%)	1 (13)	0 (0)	1

Table 1. Qualitative analyses.

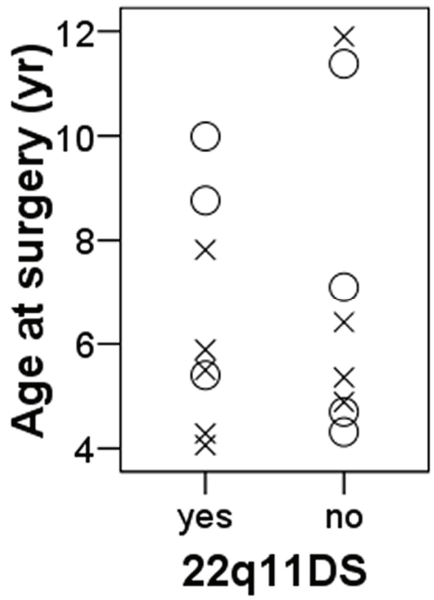

Figure 1. Group demographics. O: males, X: females.

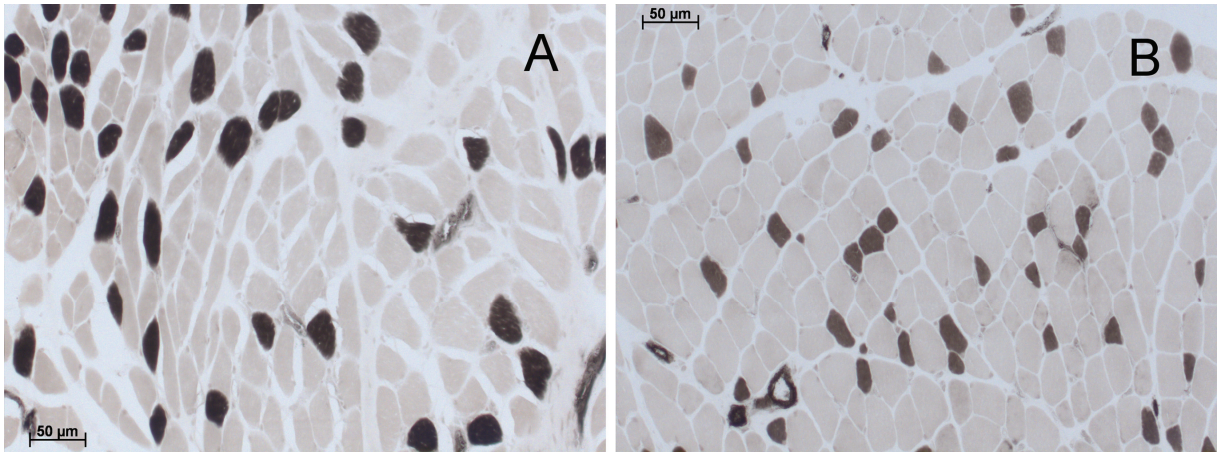

Figure 2. Histological specimens with ATPase stain at pH 4.3. A, a 5-year-old female without 22q11DS but with increased perimysial and endomysial space. B, a 10-year-old male with 22q11DS and without increased perimysial and endomysial space. Bars 50 µm.

Quantitative measurements

There was no correlation between muscle fiber diameter and age at surgery (p=0.78 for type I fibers and p=0.48 for type II fibers, Figure 3). Neither the percentage of type I fibers nor the diameters of the fiber types differed significantly between males and females or between children with and without 22q11DS (Table 2, Figure 4). All calculated fiber diameter variances were less than 250 (Table 2). For all groups, the mean diameters of type I fibers were more than 12% smaller than the mean diameters of the larger type II fibers.

Parameter	Male	Female	Mean difference (95% CI)	p	22q11DS	No 22q11DS	Mean difference (95% CI)	p
Type I fibers, % (SD)	24.8 (10.3)	30.7 (11.9)	-6 (-18, 6)	0.43	30.6 (12.3)	25.7 (10.5)	4.9 (-7, 17)	0.46
Type I fiber diameter, µm (SD)	20.6 (3.9)	18.5 (4.3)	2 (-2, 7)	0.32	19.3 (3.7)	19.6 (4.8)	-0.3 (-5, 4)	0.92
Variance	189	232			192	245		
Type II fiber diameter, µm (SD)	24.8 (2.6)	23.3 (3.3)	2 (-2, 5)	0.37	24.7 (2.8)	23.3 (3.4)	1.4 (-2, 5)	0.25
Variance	105	142			113	146		

Table 2. Quantitative analyses.

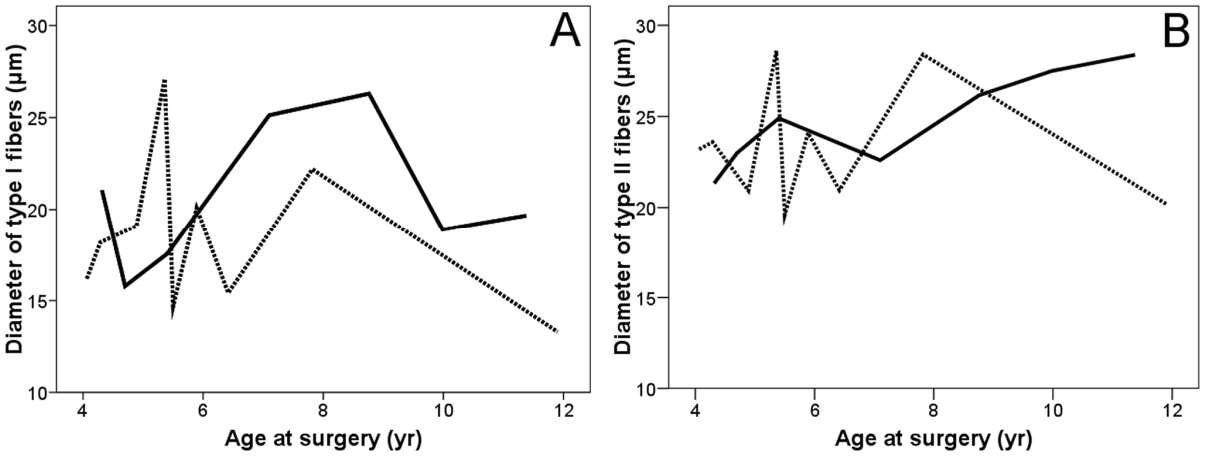

Figure 3. Mean diameters of type I (A) and type II (B) muscle fibers and age at surgery. Solid lines: males, dashed lines: females.

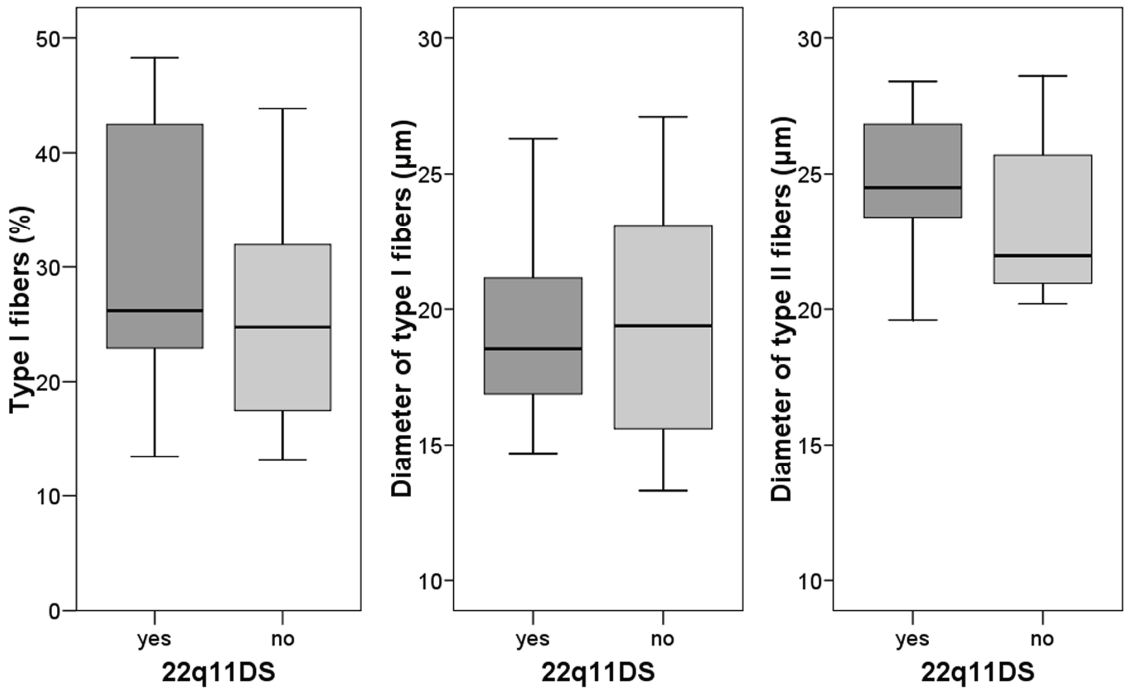

Figure 4. Muscle fiber type measurements for children with and without 22q11DS. Bands, means. Boxes, 25th-75th percentiles. Whiskers, 95% confidence intervals.

Discussion

Few studies have looked at the histology of the PCM. With the exception of specimens obtained from patients undergoing pharyngoplasty [21] or laryngectomy [43], most only study specimens from cadavers.

Morphology

Our qualitative analysis revealed no morphologic differences between PCM muscles in children with and without 22q11DS (Table 1). We found increased perimysial and endomysial space in both groups. While increased space is associated with chronic muscle damage, it is unclear whether this is also true for pharyngeal constrictors. Since it affects both groups equally, it is unlikely to be a factor in the poorer speech in children with 22qDS. Zim et al. [21] found increased endomysial space in children with 22q11DS relative to adults without the syndrome, but did not test the difference for significance. Like Zim et al. [21], we did not find any grouping by muscle fiber type, indicating the absence of innervation distubances.

Fiber type

We found 30.6% (SD 12.3) and 25.7% (SD 10.5) type I muscle fibers, respectively, in children with and without 22q11DS. Zim et al. [21] found 27.7% (SD 2.01) and 17.9% (SD 2.15) type I muscle fibers, respectively, in children with and adults without 22q11DS. The significant difference between the groups in the study by Zim et al. may not necessarily be attributed to the presence of the syndrome, but may be distorted by the unusually small percentage of type I fibers found in the adult controls (81-86 years, cadavers). Other studies on pharyngeal constrictor specimens in adults found 35% (43-77 years, live) [43], 49% (SD 9.2) (38-61 years, cadavers) [44], and 33.7% (SD 12.0) (over 50 years, cadavers) [45] type I fibers. Leese and Hopwood [45] report 20.4% (SD 8.7) type I fibers in infants (0-3 years) and 30.2% (SD 15.3) type I fibers in young adults (12-49 years). While they report no significant change with respect to age, they also report that infant muscle fibers exhibit a significantly lesser percentage of type I fibers.

Fiber diameter

Previous reports on the mean diameter of type I muscle fibers in pharyngeal constrictor muscles in adults without 22q11DS range from 26.6 to 29 μm[21,44]. In children without 22q11DS we found a mean diameter of 19.6 μm (SD 4.8). In children with 22q11DS, Zim et al. [21] found a mean diameter of 21.6 μm (SD 2.09) and we found a mean diameter of 19.3 μm (SD 3.7). It is tempting to conclude that, as with limb muscles, mean fiber diameter is related to age[46]. However, we did not find a correlation between age and diameter among children of different ages (Figure 3) and Leese and Hopwood [45] failed to find a relationship among adults of different ages. They did find a significant difference between fiber diameters in infants (0-3 years) and adults (over 12 years). Like Leese and Hopwood [45], we found no difference in fiber diameter between males and females, reflecting similar usage of the muscles by both genders.

The similar diameters of both type I and II muscle fibers in children with and without 22q11DS found in this study reflect similar strain put on this muscle by all children with VPI. Unfortunately, we did not have a control group of PCM specimens from children without VPI. Presumably, children without structural abnormalities that lead to VPI will have smaller muscle fiber diameters as they have do not have to employ the pharyngeal muscles as vigorously to close the oropharynx off from the nasopharynx.

Fiber type disproportion, reflected in a difference between the mean fiber type diameters of more than 12% of the mean diameter of the larger fiber type, is characteristic of congenital myopathies[46]. In this study, the type II fibers were more than 12% larger than the type I fibers in both children with and without 22q11DS. In the study by Zim et al. [21], the diameters of the type II fibers were also more than 12% larger than the type I fibers in children with 22q11DS, while the muscle fiber types had similar diameters in adults without 22q11DS. The disproportion is likely a result of selective type II hypertrophy rather than type I atrophy as children with VPI place extra strain on the fast type II fibers while attempting to articulate properly and preventing nasal regurgitation while swallowing.

We found greater variance in muscle fiber diameter (192 and 113) than Zim et al. [21] (97 and 77, respectively, for type I and II fibers in children with 22q11DS). Our measurements are based on more fibers per patient (171 to 200) than the study by Zim et al. [21] (64 to 113 fibers per patient). We found greater variance among children without 22q11DS (245 and 146, respectively, for type I and II fibers), but no groups had variances greater than 250, which is considered pathologic in limb muscles, but has been found in healthy palatal muscles[47].

Conclusion

Therefore, we conclude that there is no evidence of innervation or myogenic disturbances in the histologic specimens of the PCM in children with 22q11DS relative to non-syndromic counterparts. The absence of histologic deficits in the PCM muscle of patients with 22q11DS does not preclude the functional deficits manifest in the hypodynamic pharynx seen on nasendoscopy and poorer functional outcome after velopharyngoplasty. Future studies to elucidate the etiology of the pharyngeal hypotonia in 22q11DS should investigate the role of the central nervous system, such as by comparing fMRI images taken during speech. Meanwhile, unanswered etiologic and clinical questions hamper adequate management of the compromised speech understandability in patients with 22q11DS, contributing to poor social functioning and quality of life.

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CHAPTER 3

A candidate gene approach to identify modifiers of the palatal phenotype in 22q11.2 deletion syndrome patients

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Abstract

Background: Palatal anomalies are one of the identifying features of 22q11.2 deletion syndrome (22q11.2DS) affecting about one third of patients. To identify genetic variants that increase the risk of cleft or palatal anomalies in 22q11.2DS patients, we performed a candidate gene association study in 101 patients with 22q11.2DS genotyped with the Affymetrix genome-wide human SNP array 6.0.

Methods: Patients from Children's Hospital of Philadelphia, USA and Wilhelmina Children's Hospital Utrecht, The Netherlands were stratified based on palatal phenotype (overt cleft, submucosal cleft, bifid uvula). SNPs in 21 candidate genes for cleft palate were analyzed for genotype-phenotype association. In addition, TBX1 sequencing was carried out. Quality control and association analyses were conducted using the software package PLINK.

Results: Genotype and phenotype data of 101 unrelated patients (63 non-cleft subjects (62.4%), 38 cleft subjects (37.6%)) were analyzed. A Total of 39 SNPs on 10 genes demonstrated a p-value <0.05 prior to correction. The most significant SNPs were found on FGF10. However none of the SNPs remained significant after correcting for multiple testing.

Conclusion: Although these results are promising, analysis of additional samples will be required to confirm that variants in these regions influence risk for cleft palate or palatal anomalies in 22q11.2DS patients.

Introduction

The 22q11.2 deletion syndrome (22q11.2DS) is a common microdeletion syndrome that occurs in between one in 4,000 to 6,000 live births [1,2]. It encompasses the phenotypes previously known as DiGeorge syndrome, velocardiofacial syndrome, conotruncal anomaly face syndrome, many cases of the autosomal dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome (asymmetric crying facies). Patients with 22q11.2DS have a range of findings, including palatal abnormalities (overt cleft palate, submucosal cleft palate (SMCP), bifid uvula), conotruncal heart disease, characteristic facial features, immune deficiency, psychiatric problems, and learning difficulties. Structural palatal abnormalities are found in approximately one third of patients with 22q11.2DS[3]. About 16% percent have a submucosal cleft, 11% have an overt cleft, and 5% have a bifid uvula.

The 22q11.2DS is a contiguous gene deletion syndrome, which can be inherited in an autosomal dominant manner. However, over 90% of patients have a *de novo* deletion. The majority of individuals have a similar 3Mb (megabase) deletion on 22q11.2. Remarkable inter- and intra-familial clinical variability complicates genotype-phenotype correlations[4]. Possible mechanisms causing phenotypic variability may be modifier genes on the remaining allele of 22q11.2, elsewhere in the genome, epigenetic events, or chance.

Currently a large study is being carried out by the international 22q11.2 Consortium in an attempt to identify genetic modifiers of the 22q11.2DS phenotype. The study is using a genome wide single nucleotide polymorphisms (SNPs) association scan of 1,000 DNA samples. The present analysis describes a search for potential modifiers of palatal features using a candidate gene approach in 101 samples selected from the larger study. Possible association between SNPs in these candidate genes and palatal features was investigated.

Methods

Study subjects

The DNA samples described in this paper were obtained from studies concerning 22q11.2DS at the Children's Hospital of Philadelphia, USA and the Wilhelmina Children's Hospital in Utrecht, The Netherlands. The presence of the 22q11.2 deletion, prior to enrollment in this study, was confirmed using FISH or MLPA[5]. The current study was approved by the Institutional Review Board (IRB) at both centers, as well as by the Albert Einstein College of Medicine IRB in New York where genotyping was carried out (Genomics Core).

Phenotype data

Information on the presence of palatal abnormalities was obtained through database and chart review from both the Department of Clinical Genetics and the Department of Plastic Surgery at the Children's Hospital of Philadelphia and the Department of Medical Genetics at the Wilhelmina Children's Hospital in Utrecht. A total of 223 charts were reviewed (177 from Philadelphia and 46 from Utrecht). If no reliable clinical data could be obtained (i.e. when specialists did not agree, or when insufficient data was available) patients were excluded from analysis. Patients from both hospitals were stratified into two groups based on phenotype: "non-cleft" and "cleft" (overt cleft palate, submucosal cleft palate, bifid uvula).

Selection of candidate genes

One of the important genes in the typically deleted region is TBX1. Animal models of *Tbx1*, specifically those homozygous for a null allele, have shown a role of TBX1 in many of the physical anomalies that are found in 22q11.2DS, including cleft palate[6,7,8,9]. This implies that variants in the single copy of TBX1 that is present in patients with 22q11.2DS may have an effect on the development and/or severity of palatal abnormalities. Consequently, TBX1 was chosen as the first candidate gene to examine as a modifier of the palatal phenotype.

Recently, studies in mice demonstrated a possible role for Bmp antagonism and the chordin (CHRD) gene as interacting genetically with Tbx1 in mouse models[10]. As a result, CHRD was added as a candidate gene in our study. Other potential genetic modifiers outside of the deleted region in 22q11.2DS were selected based on research providing evidence of linkage or association between a genetic variant and cleft palate in humans. These were interferon regulatory factor 6 (IRF6)[11], transforming growth factor α (TGFA)[12], SATB homeobox 2 (SATB2)[13], small ubiquitin-like modifier 1 (SUMO1)[14], muscle segment homeobox (MSX1)[15,16], estrogen receptor 1 (ESR1)[17], poliovirus receptor-related 1 (PVRL1)[18], and transforming growth factor β 3 (TGFB3)[19].

Impaired fibroblast growth factor signaling has been associated with orofacial clefting[20]. Thus, the following genes were included as possible candidate genes: fibroblast growth factor 2 (FGF2), fibroblast growth factor 3 (FGF3), fibroblast growth factor 7 (FGF7), fibroblast growth factor 10 (FGF10), fibroblast growth factor receptor 1 (FGFR1), fibroblast growth factor receptor 2 (FGFR2), and fibroblast growth factor receptor 3 (FGFR3). Lastly, because studies have shown that the risk of facial clefts may be influenced by maternal folate intake [21] common SNPs in genes involved in the folate-homocysteine metabolic pathway were also investigated, namely methylene tetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), and cystathionine beta synthase (CBS).

Genotype data

Genome wide data for all subjects was acquired using the Affymetrix genome-wide human SNP array 6.0. Genotyping was carried out in the Genomics Core in the Department of Genetics of the Albert Einstein College of Medicine, New York. The array allows for the detection of 906,600 SNPs across the genome. As we used a candidate gene approach, we investigated the SNPs located in the 21 genes described above including SNPs located 5Kb on either side of each gene (for the total list of SNPs see supplement I).

To evaluate the coverage of the candidate genes that was provided by the SNPs available on the array, data was downloaded for the same gene regions in the Centre d'Etude du Polymorphisme Humain from Utah (CEU) samples from the HapMap database release 22 (http://www.hapmap.org/index/html). This data was analyzed using the Tagger procedure implemented in the Haploview software [22]. Table 1 shows the number of SNPs tested for each candidate gene, as well as the number of SNPs in the same regions in the HapMap database with a minor allele frequency >0.05 in the CEU population, the percentage of these SNPs tagged by our genotyped SNPs with an r^2>0.8, and the average r^2 value between the genotyped SNPs and the tagged SNPs.

	HapMap SNPs	Test SNPs	Captured SNPs		
Gene	N	N	N	%	r^2 mean
CBS	30	11	13	43.3%	0.99
CHRD	9	0	0	0	-
ESR1	257	65	185	72.0%	0.98
FGF2	61	17	41	67.2%	0.97
FGF3	15	7	12	80.0%	0.98
FGF7	49	9	27	55.1%	0.97
FGF10	64	21	62	96.9%	0.99
FGFR1	28	7	18	64.3%	0.95
FGFR2	72	20	34	47.2%	0.97
FGFR3	2	2	1	50.0%	1.00
IRF6	29	16	27	93.1%	0.99
MSX1	5	3	3	60.0%	0.99
MTHFR	41	6	16	39.0%	0.95
MTR	112	32	111	99.1%	0.99
MTRR	48	11	37	77.1%	0.95
PVRL1	39	15	36	92.3%	0.99
SATB2	89	24	75	84.3%	0.99

SUMO1	19	4	1	5.3%	1.00
TBX1	8	0	0	0	-
TGFA	151	43	128	84.8%	0.99
TGFB3	19	8	14	73.7%	0.97

Table 1. Cleft palate candidate genes included in our study. HapMap SNPs: number of SNPs in the HapMap release 22 in each gene including 5Kb on both sides and with a minor allele frequency >0.05 in the CEU population. Test SNPs: number of SNPs tested in this study (and included in HapMap) and with a minor allele frequency >0.05 in our population. Captured SNPs: number and percentage of HapMap SNPs tagged by the test SNPs with an r^2>0.8. r^2 mean: average r^2 between test SNPs and tagged HapMap SNPs. CEU: Centre d'Etude du Polymorphisme Humain from Utah; SNP: single nucleotide polymorphism.

Data analysis

As a means of sample quality control, all individuals with a genotype call rate (defined as the percentage of successful genotyping across the genome) less than 95% were intended to be excluded. However, as none of our samples failed this criterion, none had to be removed.

In order to implement SNP data quality control before statistical analysis, all SNPs with an individual call rate of less than 90% were removed. In addition, SNPs that failed the Hardy-Weinberg Equilibrium (HWE) test at a significance threshold of $p<0.0001$ and SNPs with a minor allele frequency below 5% were also removed. The total number of SNPs remaining after these quality control measures was 654,469. Out of these markers, the number available for each candidate gene is shown in Table 1. Two genes (CHRD and TBX1) could not be tested for association as there were few SNPs on the array for either locus and after ruling out these, no test SNPs remained after quality control.

TBX1 sequencing

As TBX1 could no longer be studied using data from the whole genome analysis, Sanger sequencing on TBX1 coding exons and evolutionary conserved non-coding regions within the gene locus was carried out on a subset of patients at the Venter Institute. The sequence of the gene included 5kb upstream and downstream of the first and last exons, respectively. The Venter Institute did the DNA sequence analysis as part of a contract from the NHLBI (http://rsng.nhlbi.nih.gov/ scripts/index.cfm). The PCR primers were designed to specifically target TBX1. Each primer pair was tailed with a universal M13 forward and reverse sequencing primer to enable subsequent sequencing. Once the regions were amplified, each PCR product was sequenced from the forward and reverse direction to provide double-stranded coverage. Sequencing was carried out with Sanger Big-Dye Terminator sequencing, and detection with capillary-based sequencing machines (Guo et al., in preparation).This generated information on SNPs in selected regions within TBX1 allowing for a more detailed analysis of the gene. The goal was to identify SNPs that alter amino acid sequence or affect splicing or a transcriptional regulatory region.

Statistical analysis

Genotyping data was exported into a text file format suitable for association analyses using the software package PLINK v1.06 [23] (http://pngu.mgh.harvard.edu/purcell/plink/). One degree-of-freedom chi-square tests of association were performed by comparing SNP allele frequencies among patients with and without palatal anomalies. Empirical p-values were calculated by permutation tests for all SNPs in each gene separately, thus providing an effective correction for multiple tests based on the number of SNPs in each gene.

Results

Study population

Genotype and phenotype data were obtained on 101 unrelated patients (Table 2). Most of these were from the Children's Hospital of Philadelphia (88 samples). The remaining 13 samples came from the Wilhelmina Children's Hospital in Utrecht. Of the 101 subjects, 38 had a form of palate anomaly (overt cleft palate, submucosal cleft palate, bifid uvula) while 63 subjects did not (for details see table 2). None of the subjects had cleft lip with or without cleft palate.

		Study Subjects		
		USA (n = 88)	NL (n = 13)	Total (n = 101)
Male		44	4	48 (47.5%)
Female		44	9	53 (52.5%)
Non-cleft phenotype		55	8	63 (62.4%)
Cleft phenotype		33	5	38 (37.6%)
	Overt cleft palate	5	1	6 (5.9%)
	Submucosal cleft palate	21	3	24 (23.8%)
	Bifid uvula	7	1	8 (7.9%)

Table 2. Characteristics of study subjects. USA: patients from Children's Hospital of Philadelphia, USA. NL: patients from Wilhelmina Children's Hospital, Utrecht, the Netherlands.

Genetic association analysis

A total of 39 SNPs on 10 genes demonstrated an asymptotic p-value < 0.05 (Table 3). These were CBS, ESR1, FGF3, FGF10, FGFR2, IRF6, MTRR, PVRL1, SATB2, and TGFA. Of these SNPs,

11 SNPs remained significant after correcting for multiple testing for the number of SNPs in each gene by means of permutation analysis. However, this significance was not retained when multiple testing for all genes was accounted for using the Bonferroni correction (threshold for experiment-wise significance $p < 0.002$).

TBX1 sequencing

TBX1 sequence data was obtained for 80 patients from the Children's Hospital of Philadelphia. Of these, 53 had a normal palate (66.3%) and 27 had a cleft phenotype (6 overt cleft, 15 SMCP, 6 bifid uvula; total 33.8%). Twelve SNPs on the TBX1 gene with an MAF>0.05 were tested for significant differences in allele frequencies between cleft and non-cleft subjects. None of the SNPs demonstrated a p-value <0.05.

Gene (band)	SNP (position)	Role	Major Allele	Minor Allele	Minor Allele Relative Frequency Cases	Minor Allele Relative Frequency Controls	Genotype Count Cases	Genotype Count Controls	OR	χ^2	P	EMP
IRF6 (1q32.2)	rs2235372 (208027059)	near-gene-3	G	A	0.25	0.12	2/15/21	0/15/48	2.47	5.81	0.02	0.06
	rs742215 (208027646)	near-gene-3	A	T	0.25	0.12	2/15/21	0/15/48	2.47	5.81	0.02	0.06
	rs17317411 (208027937)	untranslated-3	T	C	0.22	0.08	2/13/23	0/11/52	3.01	7.39	0.007	0.03
	rs17015218 (208034538)	intron	A	G	0.24	0.10	2/14/22	0/13/50	2.70	6.52	0.01	0.04
	rs17015226 (208036719)	intron	C	G	0.24	0.10	2/14/22	0/13/50	2.70	6.52	0.01	0.04
	rs6540560 (208048696)	unknown	C	T	0.24	0.10	2/14/22	0/13/50	2.70	6.52	0.01	0.04
	rs6696825 (208048995)	unknown	A	G	0.25	0.12	2/15/21	0/15/48	2.47	5.81	0.02	0.06
TGFA (2p13.3)	rs432203 (70618196)	intron	G	T	0.54	0.37	11/18/8	12/23/28	1.98	5.32	0.02	0.29
	rs377122 (70620533)	intron	C	T	0.53	0.37	11/18/9	12/23/28	1.87	4.54	0.03	0.47
SATB2 (2q33.1)	rs4673309 (199855654)	intron	A	G	0.21	0.08	1/14/23	0/10/53	3.10	7.27	0.007	0.04
	rs10497832 (199875181)	intron	G	C	0.21	0.08	1/14/23	0/10/53	3.10	7.27	0.007	0.04
	rs1374361 (199878979)	intron	C	T	0.18	0.08	1/12/25	0/10/53	2.62	4.98	0.03	0.14

	rs4675475 (199884288)	intron	G	T	0.30	0.18	2/19/17	1/21/41	1.94	3.89	0.05	0.28
	rs7569519 (199888067)	intron	A	C	0.30	0.18	2/19/17	1/21/41	1.94	3.89	0.05	0.28
	rs930616 (199894160)	intron	A	G	0.30	0.18	2/19/17	1/21/41	1.94	3.89	0.05	0.28
	rs17197938 (199908430)	intron	C	T	0.12	0.25	0/9/29	2/28/33	0.39	5.38	0.02	0.12
	rs16831370 (199939292)	intron	C	G	0.01	0.07	0/1/37	0/10/53	0.15	4.04	0.04	0.26
	rs10497836 (199996963)	intron	T	C	0.39	0.24	4/22/12	2/26/35	2.09	5.57	0.02	0.12
	rs895882 (200011260)	intron	A	G	0.39	0.24	4/22/12	2/26/35	2.09	5.57	0.02	0.12
	rs13392032 (200017738)	intron	C	G	0.29	0.17	1/20/17	3/15/45	2.04	4.27	0.04	0.21
MTRR (5p15.34)	rs17184211 (7919106)	intron	A	T	0.14	0.27	3/5/30	3/28/31	0.45	4.53	0.03	0.23
FGF10 (5p12)	rs17234541 (44344655)	intron	G	T	0.01	0.11	0/1/37	2/10/51	6.62	0.11	0.01	0.08
	rs17234079 (4497961)	intron	C	T	0.28	0.10	2/17/19	1/11/51	3.32	10.15	0.001	0.01
	rs10512852 (44429326)	unknown	T	C	0.01	0.17	0/1/37	3/16/44	0.06	12.25	0.0005	0.005
ESR1 (6q25.1)	rs1543403 (152470397)	unknown	G	C	0.59	0.44	12/21/5	10/36/17	1.82	4.14	0.04	0.63

FGFR2 (10q26.1)	rs1649202 (123230615)	intron	C	T	0.50	0.31	11/16/11	6/26/30	2.26	7.49	0.006	0.06
	rs2278202 (123233187)	intron	A	G	0.46	0.26	8/19/11	4/25/34	2.41	8.38	0.004	0.04
FGF3 (11q13.3)	rs12577891 (69332822)	near-gene-3	G	T	0.29	0.17	7/8/23	2/17/44	2.04	4.27	0.04	0.20
	rs10908228 (69336099)	intron	C	T	0.29	0.17	7/8/23	2/17/44	2.04	4.27	0.04	0.20
PVRL1 (11q23.3)	rs10790332 (119058888)	intron	T	C	0.36	0.50	5/17/16	13/37/13	0.55	4.02	0.05	0.19
	rs4936492 (119065659)	intron	G	A	0.36	0.52	5/17/16	15/35/13	0.52	4.93	0.03	0.12
	rs7950059 (119070705)	intron	C	T	0.38	0.54	6/17/15	15/38/10	0.53	4.75	0.03	0.13
	rs1467051 (119073511)	intron	G	A	0.35	0.53	5/16/16	13/39/9	0.48	6.10	0.01	0.07
	rs7945395 (119074947)	intron	A	G	0.34	0.53	6/14/18	14/39/10	0.46	6.87	0.009	0.04
	rs7945424 (119075012)	intron	G	T	0.33	0.54	5/14/17	14/40/9	0.43	7.84	0.005	0.02
	rs715849 (119080934)	intron	G	A	0.34	0.52	6/14/18	13/40/10	0.47	6.31	0.01	0.06
CBS (21q22.3)	rs2839631 (43343155)	unknown	C	T	0.46	0.33	7/21/10	5/31/27	1.77	3.69	0.05	0.22
	rs9325622 (43352770)	intron	G	A	0.46	0.32	7/21/10	5/30/28	1.84	4.16	0.04	0.19

	rs1789953 (43356005)	intron	T	C	0.07	0.18	0/5/33	2/19/42	0.32	5.41	0.02	0.10

Table 3. Results of association analysis of candidate genes with nominal p values < 0.05. SNP: single nucleotide polymorphism. Major allele: most frequent in sample. Minor allele: least frequent in sample. OR: odds ratio. χ^2: chi square statistic. P: nominal p value. EMP: empirical p value after permutation analysis.

Discussion

This report rules out common SNPs in the most promising candidate genes as being major modifiers of the palatal phenotype in 22q11.2DS. It does provide tentative evidence for modest modifiers and suggests a relationship between a number of cleft palate candidate genes and the development of palatal anomalies in 22q11.2DS. The gene with the most significant SNPs associated with cleft palate in our data set is FGF10. A number of studies have shown a role for fibroblast growth factors including FGF10 in orofacial cleft development[20].

In embryology, the formation of the pharyngeal arches plays a central role in the development of the face and neck. The genetic regulation of this craniofacial myogenesis, however, remains relatively unknown[24]. A recent study by Kelly et al [25] demonstrated that *Tbx1* is an important regulator in the onset of branchiomeric myogenesis and pharyngeal muscle development in the mouse. It is hypothesized that *Tbx1* is required for transcriptional activation of myogenic determination genes as it showed that *Tbx1* regulates the expression of *Fgf10* in the core of the first pharyngeal arch. Mutations in *Tbx1* resulted in down-regulation of *Fgf10* expression which affected the patterning of cells in the mandibular arch and thus resulted in defects in branchiomeric myogenesis in mice[25]. Another study, by Rice and colleagues showed that FGF10 is crucial in mediating tissue-tissue interactions during palate development[26]. Mice lacking *Fgf10* did show initial palatal shelf buds but they did not undergo palatal extension and growth.

These studies of animal models illustrate both the important role of FGF10 in palate development and the important interaction between FGF10 and TBX1. Unfortunately, the SNPs that were tested in FGF10 in the current report did not retain significance after correction for multiple testing. This may be a due to a number of possible limitations, such as the number of selected SNPs being too low to achieve full gene coverage and/or the small number of analyzed patients. In order to gain more information on TBX1 it was sequenced in a subgroup of our subjects with sufficient DNA material. Unfortunately, no significant SNPs were found. Again, this may be due to low subject numbers.

Though no significant SNPs were identified, this study offers preliminary results from the larger study that is currently being carried out by the International 22q11.2 Consortium. The 22q11.2DS phenotype can be extremely variable and thus presents a challenge for genotype phenotype association studies. As more whole genome data of 22q11.2DS patients becomes available, true correlations are likely to gain statistical significance.

In summary, in this research report we investigated association of development of palatal anomaly in 22q11.2DS with variants in known cleft palate genes. Despite the small sample size, some variants showed nominal significance and might act as moderate genetic modifiers. However, although 11 SNPs retained statistical significance after correcting for the number of SNPs tested in each individual gene, none of these were significant after correcting for the total number of genes tested. As this project is part of a larger study being performed by the International 22q11.2 Consortium, additional DNA samples should provide more data in the future. The results from these additional samples will be required to confirm the findings in this report.

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CHAPTER 4

Outcome of velopharyngoplasty in patients with velocardiofacial syndrome

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Abstract

Background: Surgical correction of velopharyngeal insufficiency (VPI) in patients with velocardiofacial syndrome (VCFS) is challenging, requiring a high rate of revision. Here, the management of VPI in patients with VCFS is reviewed, and outcome is compared to a non-VCFS group.

Methods: 25 patients with VCFS (16 girls; 9 boys) underwent palatal lengthening for VPI between 1986 and 2001. The mean age at surgery was 6,4 years. Revision was defined as the need for secondary sphincter pharyngoplasty as determined by speech investigation, nasal endoscopy, and acoustic nasometry. A comparison was made to a control group comprised of a randomised group of non-VCFS patients who underwent palatal lengthening for VPI (32 patients; 10 girls and 22 boys).

Results: In the VCFS group, 16% (4/25) of the patients required surgical revision. These patients were slightly older at the time of primary surgery (6 versus 5,5 years). In the control group, no patients required revision. Preoperative speech analysis showed a more pronounced VPI in the VCFS group. Outcomes of endoscopy and speech hypernasality improved significantly more in the control group. Improvement in the results of acoustic nasometry did not differ significantly between the two groups.

Conclusion: Treatment of VPI using palatal lengthening in children with VCFS is both safe and effective. The discrepancy in improvement between the speech analysis and the nasal endoscopy results within the VCFS group indicates that mechanical improvement does not necessarily correspond to an improvement in speech. This emphasizes the complexity of speech disorders found in VCFS.

Introduction

Hypernasality is a feature of velopharyngeal insufficiency (VPI). VPI is the inability to completely close the velopharyngeal port during speech production. VPI can result from a variety of causes; the most common cause being an overt or submucous cleft of the secondary palate [1]. In patients with a cleft palate, insufficient tissue, insufficient muscular activity, or scar tissue may impair velopharyngeal closure.

If hypernasality persists after primary repair of the cleft palate a pharyngoplasty can be done to normalize resonance during speech production. In our hospital, we routinely use a palatal lengthening procedure for patients with velopharyngeal insufficiency [2, 3]. Here, palatal lengthening is achieved by pushback with a pharyngeal flap using only mucosal flaps instead of full thickness mucoperiosteal flaps for the oral lining of the defect [3].By preserving the periosteum and the palatine arteries, vascularization of the hard palate is maintained and bone is not exposed, avoiding potential detrimental scar formation overlying the hard palate, which may affect normal outgrowth of the maxilla.The level of improvement after surgical intervention varies between patients. One of the explanations for this individual variation is the existence of a conjoint syndromal abnormality like the velocardiofacial syndrome (VCFS).

VCFS has also been called DiGeorge Syndrome (DGS), Shprintzen Syndrome or Conotrucal Anomaly Face Syndrome (CTAF). VCFS is an autosomal dominant disorder caused by the 22q11.2 deletion and is subsequently often referred to as 22q11.2 deletion syndrome. The incidence is approximately 1 per 4000 live births [4]. It has a highly variable phenotype and has multiple anomalies such as heart malformations, typical facial characteristics, developmental difficulties, a cleft palate, velopharyngeal insufficiency and a characteristic pattern of hypernasal speech [5, 6, 7, 8]. In patients with VCFS, pharyngeal muscle hypotonia is present in approximately 75% of patients contributing to hypernasality [9].

In this report, our experiences in functional outcome after palatal lengthening velopharyngoplasty in patients with VCFS are described and compared to a group of patients without VCFS. In addition, the clinical features and pre- and postoperative speech analyses of patients with and without VCFS are reviewed.

Methods

Demographics

Records of the patients who underwent palatal lengthening for velopharyngeal insufficiency in the period 1986-2001 in our hospital were reviewed. The VCFS group consisted of 25 consecutively treated patients; 15 girls and 10 boys, with a mean age at surgery of 6,4 years (Table 1). Mean follow-up was 5 years. Comparisons were made to 32 non-syndromic patients who also underwent palatal lengthening surgery to correct hypernasality (submucous cleft palate n=14, congenital short velum n=5, cleft palate n=6, unilateral complete cleft n=6, bilateral complete cleft n=1). The control group comprised of 10 girls and 22 boys with a mean age at surgery of 7.4 years (Table 1). All 57 patients had undergone a fluorescence in situ hybridization (FISH) test to either confirm or deny the presence of the 22q11.2 deletion that causes VCFS.

	Girls	Boys	Total
VCFS	16	9	25
Non VCFS	9	23	32
Total	25	32	57

Table 1. Description study group.

Analysis of velopharyngeal function

All patients were subjected to pre- and postoperative testing of velopharyngeal function by a phoniatrician and a speech pathologist. Screening included a perceptual speech investigation, nasal endoscopy, and acoustic nasometry. Perceptual speech investigation was performed using Eurocleft sentences which are designed in such way that every sentence contains two explosive consonants in the same position in five languages, including Dutch [10]. The degree of hypernasality during speech was categorized as always, sometimes, and never. Nasal endoscopy was done using a Pentax 2,3 mm flexible endoscope (Pentac Corp., Tokyo, Japan) and documented on videotape (Sony U-matic VCR V07630). Patients were asked to repeat high-

and low pressure oral and nasal loaded speech. Overall velopharyngeal function, including velar elevation, lateral wall movement, and closure pattern was rated as always, most of the time, sometimes, and never. Acoustic nasometry data was obtained using a NasalView® instrument in combination with "Dr. Speech for windows" [11, 12]. Nasalance scores of nasal and oral sounds were registered both pre- and postoperatively.

Statistical analysis

The results of all investigations were statistically analysed. For the acoustic nasometry, a paired samples t-test and an independent sample t-test was used. The results of nasoendoscopy, the hypernasality speech tests, and the velum function tests were analysed with a Wilcoxon signed ranks test and a Mann-Whitney test. For the entire statistic analysis, we used complete result-couples. Statistical significance was defined as $P < 0.05$.

Results

Primary Success of Surgery

In the VCFS group, 16% (4/25) of the patients required surgical revision. For these patients, the average age at initial surgery was slightly higher; 5.5 versus 6.0 years. Average time interval to revision was 6.7 years (ranging from 3 to 8 years). This delay was due to a variety of causes, including loss to follow up (1 patient), reluctance of parents for reoperation (1 patient) and hesitation of the surgeon about the efficacy of reoperation in VCF patients with poor palatal mobility (2 patients). Revision was done using a sphincter pharyngoplasty [13, 14]. This technique yielded a clear anatomic improvement in 3 of 4 patients (full closure achieved during nasoendoscopy) combined with an audible improvement of speech, but speech normalised in only one of them. The one patient whose speech did not improve after revision showed minimal to absent activity of the velum and pharyngeal wall. In the control group none of the patients required revision.

Perceptual Speech analysis (Table 2 A, B)

24 of 25 patients (96%) in the VCFS group were preoperatively determined to be in the "always" category of speech hypernasality. The remaining patient was rated as "sometimes". Post-operatively, 58% of patients (15/25) did not show a decrease in hypernasality. The remaining 42% of VCFS patients did show an audible improvement , but none of them achieved normal speech after the operation.

In the control group, 91% of patients (29/32) displayed hypernasal speech pre-operatively categorized as "always". The remaining three patients (9%) were judged to be in the "sometimes" hypernasal category. 78% of patients showed improvement post-operatively and 50% of patients normalized completely. Multivariate analysis confirmed the significant improvement in hypernasality following primary surgery both in the study groups ($p=0.001$ for the VCFS group; $p<0.0001$ for the control group) and between the study groups ($p < 0.001$).

	Before	After
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		

Table 2 A. Degree of hypernasality in the VCFS group before and after pharyngoplasty. Red: always; yellow: sometimes; green: never; x: no data available

	Before	After
1		
2		
3		
4		
5	X	X
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31		
32		

Table 2 B. Degree of hypernasality in the control group before and after pharyngoplasty. Red: always; yellow: sometimes; green: never; x: no data available

Nasal endoscopy (Table 3 A, B)

Not all patients cooperated sufficiently to get complete data pre- and postoperatively. For statistical analysis solely the complete data couples were used. As a result, the VCFS group was reduced to 16 patients and the control group to 25 patients. Multivariate analysis demonstrated improvement of nasopharyngeal closure following surgery in both groups (VCFS group, $p=0.002$; control group, $p<0.0001$) and between the VCFS and non-VCFS groups respectively ($p<0.007$).

	Before	After
1		
2		
3		X
4		
5		
6	X	X
7		
8		
9	X	X
10		
11	X	X
12		
13	X	X
14		
15	X	
16		
17	X	X
18	X	
19		
20	X	
21		
22		
23		X
24		
25		

Table 3 A. Closure pattern in the VCFS group before and after pharyngoplasty. Red: never; orange: sometimes; yellow: often; green: always. x: no data available

	Before	After
1	X	X
2	X	X
3		
4		
5	X	X
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18	X	X
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31		
32		

Table 3 B. Closure pattern in the control group before and after pharyngoplasty. Red: never; orange: sometimes; yellow: often; green: always; x: no data available

Acoustic nasometry (Supplement II)

In the VCFS group, 10 patients were tested pre- and postoperatively. In the control group, 28 patients were tested. There was significant improvement in both groups (standard text $p<0.01$, denasal text $p<0.0001$ for both groups). An independent samples t-test, comparing the degree of improvement between the VCFS and non-VCFS groups, demonstrated no significant difference.

Discussion

Speech and language problems are among the most common characteristics of VCFS [15]. The treatment of velopharyngeal insufficiency in patients with VCF is particularly challenging. VCFS children have several intrinsic anatomical and physiological characteristics that may influence velopharyngeal closure. In addition to the cleft palate, several factors may influence VPI. Pharyngeal hypotonia, platybasia (resulting in an increased palate-to-posterior pharyngeal wall distance), unilateral vocal fold paralysis, and adenoid hypoplasia may contribute to the VPI. With the palatal lengthening procedure used in our hospital, speech evaluation and analysis showed a satisfactory result in 84% of VCFS patients. After revision, all but one patient showed further improvement in speech. All patients in the control group demonstrated improvement in speech and none required revision.

Golding-Kushner suggests that communication skills in children with VCF may be syndrome specific [15]. Nearly 98% of patients with VCF have developmental delay [15]. When compared to the patients in our control group, the VCFS patients had trouble cooperating with and completing the different pre- and postoperative tests, reducing the available complete data scores. This seems to be inherent to the psycho-social development and personality characteristics of VCFS patients.

This discrepancy in revision rate in our study may be explained by the fact that preoperative speech analysis showed more pronounced velopharyngeal insufficiency in the VCFS group. The results of speech evaluations and nasal endoscopy demonstrated significant discrepancies between VCFS and controls. Both before and after surgery non-VCFS patients performed better. Post-operatively, the control group improved to a higher level of velopharyngeal closure, decreased hypernasality, and improved velum function than the VCFS group. Our nasometry numbers indicate that the degree of improvement did not differ significantly between both groups, but because the severity of the hypernasality was more pronounced in the VCFS group, the improvement was often not enough to fully alleviate VPI in the VCFS group.

Therefore, a mechanical improvement in the VCFS group may not necessarily result in a normalization of speech. Functional brain imaging has provided insight into the abnormal neurological mechanisms underlying the mathematical and speech problems seen in VCFS [16]. This illustrates the complexity of the speech disorders found in VCFS. In VCFS, many other factors are presumed to play a role in the outcome after surgery, including abnormal levator muscle anatomy, abnormal dimensions of the oropharynx, abnormal brain development and psycho-social disorders [9, 17, 18, 19]. Reoperation rate was low despite the fact that in the majority of the VCFS group normal speech at time of the measurement in the postoperative period was not achieved. This indicates that the final outcome is sufficient for the children to perform at school and have good social contacts. We monitor this closely and school and / or social problems due to incomprehensible speech are an indication to perform additional surgery in children with VCFS in our unit. In order to investigate this further the authors intend to undertake a long term follow up study of these patients.

In our experience, the beneficial effects of speech correcting surgery in the VCFS population are best evaluated one year after velopharyngoplasty. Patients with VCFS generally need such a time period to adjust and correct their speech pattern and techniques. In our experience this adjustment to the new anatomical situation after surgery is markedly slower compared to the non VCFS patients. At present we can only speculate about the cause of this slower adjustment. The lower average intelligence of the VCFS population compared to a normal population is a possible factor of importance. One of the limitations of our study was that patients were not matched for IQ. However, other studies do not mention the slower adjustment in the VCFS population [20]. In the future we intend to report on the long term effects of palatal lengthening on speech in VCFS patients, but good prospective randomised studies will be required to get the final answer.

An important benefit of the palatal lengthening technique as used in our hospital is the fact that a sphincter pharyngoplasty can still be carried out in case the initial result is unsatisfactory. This is especially useful in cases of asymmetry, as reported in VCFS patients

[21]. In these cases, a unilateral or bilateral sphincter pharyngoplasty may provide the finishing touch. This was necessary in 4 patients in our VCFS group, yielding a re-operation rate of 16%. This revision rate compares favourably with the results of Losken et al [20]. These authors compared the results of sphincter pharyngoplasty for the treatment of VPI in patients with VCFS and a control group of patients without VCFS, similar to our report. They found - as we did - that VCFS patients had significantly less favourable results with a relatively high revision rate of 22%. However, the data collection in their study was different from ours precluding a detailed comparison between their and our VCFS outcomes.

In 2004, Mehendale et al also reported on the management of VPI in patients with velocardiofacial syndrome [21]. Their study lacks the comparison with a control group. They advocate a staged approach, repairing and retropositioning the muscle first in cases of anterior levators or submucous cleft palate. If this yielded insufficient effect a Hynes pharyngoplasty was carried out. It is evident from their study that normal speech cannot always be achieved in VCFS patients, even after a staged procedure.

Our study has several shortcomings. It is a retrospective study and includes several limitations such as possible loss of patients for follow-up, selection bias and our inability to adequately control for additional variables. The intent of our analysis was to evaluate our surgery method and investigate whether this is a safe and reliable method to improve speech problems in VCFS. Formulating a hard conclusion from our results is further hampered by the fact that outcome of VPI surgery generally is not measured and reported in a uniform way. This makes comparison between studies very difficult, if not impossible. The relatively scarce literature reporting about outcome of VPI surgery in the VCFS population shares the same limitation. Based on the available data we conclude that a palatal lengthening procedure to treat VPI in patients with VCFS is as successful as any other technique reported. The procedure is certainly safe in VCFS patients and it has the advantage that a sphincter pharyngoplasty (Orticochea or Hynes technique) is still possible. As such, our approach resembles the philosophy of others like Mehendale et al [21] in the treatment of VCFS patients, not burning any bridges and going step by step, accepting the fact that more than one surgery may be necessary to achieve an optimal result in the treatment of VPI.

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CHAPTER 5

Velopharyngeal dysfunction and 22q11.2 deletion syndrome: a longitudinal study of functional outcome and preoperative prognostic factors

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Abstract

Objective: To describe the effect of time after velopharyngoplasty on outcome and search for preoperative prognostic factors for residual hypernasality in patients with 22q11.2 deletion syndrome (22q11.2DS).

Design: Retrospective chart review.

Setting: Tertiary hospital.

Patients: Patients with 22q11.2DS and velopharyngeal dysfunction (VPD) who underwent a primary (modified) Honig velopharyngoplasty between 1989 and 2009.

Main outcome measures: Clinically obtained perceptual and instrumental measurements of resonance, nasalance, and understandability before and after velopharyngoplasty.

Results: Data was available for 44 of 54 patients (81% follow-up), with a mean follow-up time of 7.0 years (range 1.0-19.4 years). During follow-up, 24 (55%) patients attained normal resonance and 20 (45%) had residual hypernasality or underwent revision surgery. Mean postoperative nasalance and understandability scores were closer to normal values than mean preoperative scores (2.0 vs 5.5 SD for the normal passage, 1.3 vs 8.1 SD for the non-nasal passage, and 2.3 vs 4.1 understandability). Serial measurements revealed that hypernasality only resolved on average five years after surgery, and three patients' whose resonance initially normalized later relapsed to hypernasality. Gender, age at surgery, lateral pharyngeal wall adduction, velar elevation, presence of a palatal defect, previous intravelar veloplasty, nasalance, understandability, adenoidectomy, hearing loss, and IQ were not able to predict poor outcome following primary velopharyngoplasty (all $p>0.05$).

Conclusion: In this chart review of patients with 22q11.2DS and VPD, residual hypernasality persisted in many patients after velopharyngoplasty. None of the preoperative factors that were studied had prognostic value for the outcome.

Introduction

The 22q11.2 deletion syndrome (22q11.2DS) is the most frequent human microdeletion syndrome [1]. The frequency is estimated around 1 in 4000 [2] but may be as high as 1 in 2000 surviving newborns[3]. Over 180 clinical features, including every organ system, have been associated with the deletion[4].

One of the most common clinical features is velopharyngeal dysfunction (VPD), affecting 27-92% of children with 22q11.2DS[5]. The 22q11.2DS is the most common diagnosis in patients with VPD of unknown cause[6]. VPD is the incomplete closure of the velopharyngeal valve which normally separates the oral and nasal cavities, resulting in nasal regurgitation during feeding, frequent otitis media, and hypernasal speech[7]. In 22q11.2DS, the etiology is related to structural abnormalities such as palatal anomalies in 34% of patients [7], but may also be the corollary of cranial nerve dysfunction[8]. Surgeons aim to correct VPD by improving the velopharyngeal closure. This can be done by lengthening the palate, mobilising a pharyngeal flap that spans the center of the gap but retains lateral ports, or rotating lateral flaps to augment the sphincter[9]. In general, the speech outcome after surgery has been reported to be worse in patients with 22q11.2DS than in patients without the syndrome [10-16], but some patients with 22q11.2DS fare as well as their non-syndromic counterparts after surgery [17-22]. Naturally, parents are interested to know whether their child will benefit from surgery. However, prognostic factors remain elusive [14].

All postoperative outcome studies to date have mean follow-up periods of less than 5 years. This report includes an analysis of the functional outcome after a follow-up up to 19 years after primary velopharyngoplasty in patients with 22q11.2DS. To do this, a group of patients previously reported on [16] was augmented with more recent patients. The purpose of this study was to describe the effect of time on functional outcome and search for preoperative prognostic factors for residual perceptual hypernasality or the need for surgical revision following velopharyngoplasty.

Methods

Patients

Postoperative functional outcome was inventoried from the medical records of patients with FISH-confirmed 22q11.2DS who underwent a primary (modified) Honig velopharyngoplasty for VPD between 1989 and 2009 in our tertiary hospital. These surgeries include both palatal lengthening by pushback and raising a superiorly based pharyngeal flap from the posterior pharyngeal wall. The lateral edges of the flap curl under causing it to tube. While the conventional Honig velopharyngoplasty uses full thickness mucoperiosteal flaps for the oral lining of the defect, the modified technique uses only mucosal flaps[23]. Only patients for whom resonance was measured preoperatively and at least one year postoperatively was available were included since resonance takes at least a year to stabilize after surgery[16, 22, 24, 25, 26]. The outcome of a subgroup of 25 patients was previously reported after a mean follow-up time of five years[16]. These patients were invited to return for long term follow-up assessment at the outpatient clinic.

Between 1989 and 2009, 54 patients with 22q11.2DS underwent a primary (modified) Honig velopharyngoplasty at our institution. All patients had intensive speech therapy before and after surgery. Assessments of resonance both preoperatively and at least one year after primary velopharyngoplasty were available for 44 of these patients (81% follow-up). One patient was excluded because she did not speak preoperatively, precluding preoperative resonance assessment. The other nine patients were excluded because they only returned for follow-up assessments within one year after primary velopharyngoplasty. No reasons were recorded for discontinued follow-up. Patient demographics are listed in Table 1. As indicated by the inclusion criteria, the minimum postoperative follow-up time to speech assessment was one year. The maximum follow-up time was 19.4 years after primary velopharyngoplasty with a mean of 7.0 years. Intravelar veloplasty constitutes the anatomic dissection and repositioning of the velar muscles. On all but one occasion when revision surgery was performed, this was for residual hypernasal speech. The exception was one patient without residual hypernasality whose speech continued to be perceptually bothersome after over eight years of speech therapy after primary pharyngeal flap surgery. The first and second revisions were performed an average of 6.2 years

(range 1.5-11.0 years) and 8.6 years (range 5.1-12.1 years) after primary velopharyngoplasty, respectively. Based on patient histories, no patients suffered from obstructive sleep apnea postoperatively.

Outcome measures

By surgically creating an autologous obturator between the oro- and nasopharynx, treatment for VPD most directly aims to ameliorate hypernasality. Resonance was tested during live assessment preoperatively and postoperatively at varying follow-up times using standardized passages. The 'normal' passage has a proportion of nasal sounds representative for Dutch language similar to the Rainbow passage. The 'non-nasal' passage is similar to the Zoo passage in that it has no nasal sounds[27].

Perception is the gold standard of speech assessment[28]. Speech pathologists graded hypernasality using the three-point-scale used by the Dutch Association for Cleft and Craniofacial Anomalies[29,30]. A score of 1 denotes normal resonance on vowels, a score of 2 denotes hypernasality on vowels, and a score of 3 denotes hypernasality on vowels and approximants. Documentation in patient charts, however, was inconsistent, often only stating whether resonance was normal or hypernasal. Therefore, only normal or hypernasal resonance was inventoried for this chart review.

While the perceptual speech test used by the Dutch Cleft Palate Association has not officially been tested for validity, some believe there is poor inter- and intrarater reliability for the perceptual assessment of hypernasality. Therefore, the speech pathologists at our center frequently measured nasalance instrumentally with the Nasometer 6200 (Kay Elemetrics) until 1999, and the NasalView (Tiger DRS Electronics) from 2000 onwards. These measurements were inventoried as secondary outcome measures. As these machines have different calibrations[27, 31], the nasalance percentage scores could not be compared directly. Instead, the standard deviations were calculated for the percentage scores. Values within two standard deviations (SDs) greater than or less than the normal score were considered to be within the

normal range[27, 31]. Occasionally, when perceptual resonance was normal, speech pathologists forewent instrumental measurements.

Speech understandability is less directly influenced by surgery as it a sum of many speech components besides resonance, including articulation and voice quality. It was inventoried for this study as it is socially important[32]. Based on live conversational speech, a speech pathologist graded the understandability together with the patients and their parents using the five-point-scale used by the Dutch Association for Cleft and Craniofacial Anomalies [29, 30] (Table 2). A score of 1 indicated normal speech and a score of 4 or 5 indicated poor speech understandability.

As aforementioned, resonance takes at least a year to stabilize after surgery [16, 22,24,25,26]. Lipson et al. [32] found improvement occurred up to four years after surgery. To examine whether speech continues to change, serial assessments in patients with multiple assessments were compared. As this was a retrospective study, patients had not been invited for measurements at regular intervals. Limited data precluded statistical analysis; descriptive analyses are presented.

Poor outcome after primary pharyngeal flap surgery was defined as residual perceptual hypernasality or the need for surgical revision. Based on studies including both syndromic and non-syndromic patients with VPD, the following potential preoperative prognostic factors for poor postoperative outcome were analyzed in the 22q11.2DS population: male gender [33], age >7 years at surgery [34], poor or moderate lateral pharyngeal wall adduction [35-38], poor or moderate velar elevation, presence of a palatal defect [39], previous intravelar veloplasty, adenoidectomy, hearing loss of at least 40dB in both ears, IQ <70 [40], poor understandability, and high nasalance scores[15]. Lateral pharyngeal wall adduction and velar elevation had been assessed during nasal endoscopy with a Pentax 2.3-mm flexible endoscope. Motion had been categorized as either poor, moderate, or good. IQ was measured using the age-appropriate WPPSI-R, SON-R, or WISC-III scales.

Statistical analysis

The independent T-test was used to compare the mean follow-up time to resolution of hypernasality, mean age at primary velopharyngoplasty, mean preoperative nasalance, and mean preoperative understandability between the group that attained normal resonance and the group that had residual hypernasality or underwent revision surgery. A two-tailed Spearman correlation was used to quantify the correlation between resonance and nasalance, and between resonance and understandability. The values of the potential prognostic factors for predicting poor outcome were tested using the Fisher exact test. Only complete data pairs were used.

Results

Pre- and postoperative speech assessments

Hypernasal resonance is the indication for velopharyngoplasty. After primary velopharyngoplasty, 24 (55%) patients attained normal resonance and 20 (45%) had residual hypernasality or underwent revision surgery (Figure 1). The follow-up time to either outcome did not differ significantly between the groups (5.2 years (range 1.1-17.4) vs 4.9 years (range 1.0-15.5), p = 0.80), neither did the age at primary velopharyngoplasty (5.9 years (range 3.4-10.0) vs 6.0 years (range 3.4-13.9), p = 0.88). Normal resonance was not limited to those who had been followed for at least 5 years. Three of the patients who attained normal resonance relapsed to hypernasality on average 2.0 years after achieving normal resonance. Of the eight who underwent revision surgery, two went on to attain normal resonance. At maximum follow-up, 21 (48%) had residual hypernasality, including six who remained hypernasal after revision surgery.

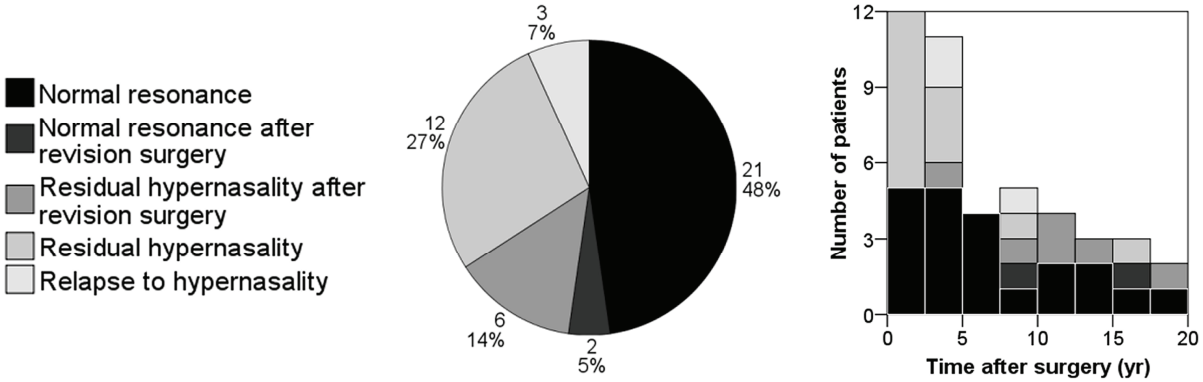

Figure 1. Resonance at maximum follow-up, including the total number of patients with each outcome and stratification by follow-up time.

Given the retrospective nature of this cohort study, although resonance assessments were available for all patients based on the inclusion criteria, nasalance and understandability scores were not. The mean nasalance and understandability scores at maximum follow-up showed an improvement relative to the mean preoperative scores (Table 3). Resonance was more highly correlated to the nasalance scores (0.67 for the normal passage, and 0.71 for the non-nasal passage) than to understandability (0.48) (Figure 2).

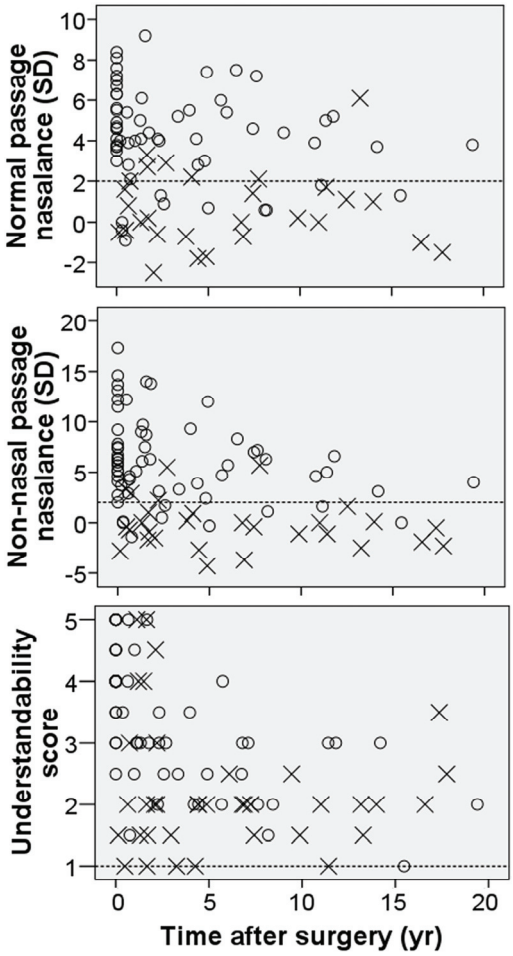

Figure 2. Nasalance, understandability, and resonance measured preoperatively (time after surgery 0) and at varying times after velopharyngoplasty. Dashed lines: upper limits of normal nasalance and understandability scores. X, normal resonance; O, hypernasal.

Prognostic factors

Again, as this was a retrospective study, not all of the potential preoperative prognostic factors had been measured or recorded for all patients. None of the factors tested were prognostic for poor outcome following primary velopharyngoplasty (Table 4). The group of patients with poor outcome did not differ from the group that attained normal resonance regarding the preoperative nasalance while reading or repeating the normal passage (5.9 vs 5.0 SD. p = 0.24) or the non-nasal passage (9.1 vs 6.9 SD, p = 0.23), nor understandability (4.0 vs 4.1, p = 0.55).

Discussion

Speech generation and perception are complex, involving cognition, language, and voice. Understanding and managing speech problems in patients with 22q11.2DS is especially challenging because of the gamut of clinical findings associated with the syndrome and the heterogeneous presentation among patients[16]. Anatomically, palatal defects, adenoid hypoplasia [42], and platybasia [42] enlarge the pharyngeal gap, and medially placed internal carotid arteries call for extra caution during surgery[43]. Comorbidities such as cardiac anomalies and immune deficiencies may delay or preclude surgery. Additionally, muscle hypotonia [44, 45, 46], asymmetric palatal elevation[25, 47, 48, 49], hearing disorders[47], schizophrenia[50], and learning disabilities [5, 51 52] may hamper speech therapy.

The effect of time on functional outcome

Rouillon et al.[22] showed that postoperative speech in patients with 22q11.2DS was inferior to that of patients without 22q11.2DS at 9 months, but equal at 24 months. The authors postulated that this delayed improvement can be attributed to psychomotor retardation or acquisition difficulties which are common in 22q11.2DS[5, 51,52]. As was described by Widdershoven et al. [16], our experience confirms that the adjustment to the new anatomic situation after surgery is markedly slower in patients with 22q11.2DS than in patients without 22q11.2DS. Witt et al [53] found that perceptual speech scores at ages 6 and 12 years after cleft palate repair are stable in children without 22q11.2DS. Following pharyngeal flap surgery in children without 22q11.2DS, Riski et al.[34] report that the percentage of patients with acceptable resonance remained consistent 2 and 5 years after, and Cable et al.[54] found that overall resonance continued to be adequate up to 14 years after pharyngeal flap surgery. Given the complex nature of the speech problems in 22q11.2DS, we were curious whether postoperative speech outcome changes over time in this population. All postoperative outcome studies in patients with 22q11.2DS to date have mean follow-up periods of less than 5 years. In this report, we present the functional outcome up to 19 years after primary pharyngeal flap velopharyngoplasty.

Previous studies only note that resonance takes at least a year to stabilize after surgery[16, 24, 25]. In this study we show that speech continues to evolve as patients age. In patients whose resonance normalized, this only occurred an average of 5 years after primary velopharyngoplasty. Perhaps the patients with residual hypernasality who were followed for less than 5 years will eventually attain normal resonance and were therefore erroneously categorized as having a poor outcome. Once normal resonance was attained, some patients relapsed to hypernasal speech. These continuing changes in resonance make conclusions about postoperative outcome questionable.

The upper limits of the ranges of follow-up times around each of the calculated means may illustrate the marked phenotypic heterogeneity in 22q11.2DS, or be artifacts of this retrospective study in which patients had not been assessed at regular intervals after surgery. For example, the patient who only attained normal resonance 17.4 years after primary velopharyngoplasty had been dismissed from clinical follow-up 5.7 years after surgery, at which point his resonance was still hypernasal. When he was invited to return for re-assessment, his resonance was normal. It is unclear when his resonance normalized. Patients with poorer speech return more frequently for follow-up consultations and measurements, introducing a selection bias. A prospective study where all patients are measured at regular intervals should avoid selection bias and ensure sufficient data pairs for statistical analysis.

Outcome measures

While perceptual speech is the gold standard for assessing the success of a velopharyngoplasty[28], there is no standardized reporting system. Henningsson et al.[55] have suggested a system that including the parameters hypernasality, hyponasality, audible nasal air emission and/or nasal turbulence, voice disorder, consonant production errors, understandability, and acceptance. They suggest continued usage of local measures with mapping to a universal scale to allow comparison of outcomes between centers. The speech test developed and used by the Dutch Association for Cleft and Craniofacial Anomalies measures all parameters except voice disorder, but has not officially been tested for validity nor

reliability. The speech test uses a three-point scale to rate resonance differentiating between hypernasality on vowels or consonants. However, due to inconsistent reporting in the charts, in this study resonance was recorded as either normal or hypernasal. Dichotomous scales generally yield higher agreement and reliability, but this made it impossible to grade improvements in resonance other than complete normalization, underestimating the effect of velopharyngoplasty in partially correcting hypernasal resonance.

Along with perceptual speech rated by a speech pathologist, various surrogate outcome measures are used to assess speech and the success of a velopharyngoplasty. For example, revision rates are easy to measure. However, they are not always indicative of success[15]: sometimes the surgeon gauges that further surgery will not be beneficial, and sometimes patients are satisfied with improved speech and therefore do not opt for further surgery to optimize speech. Patient satisfaction, another outcome measure, is more subjective than perceptual speech assessed by a speech pathologist. Some find that hyponasality causes less social stigmatization than hypernasality[56]. Nasendoscopic velopharyngeal closure is difficult to assess objectively[56, 57, 58]. Objective measures such as and nasalance measured with the Nasometer or NasalView are often reported, however the correlation with perceptual resonance varies from 0.31 to 0.74, limiting its use to measuring the degree of hypernasality once hypernasality has been diagnosed perceptually[60, 61] (Figure 2).

Understandability, which is perhaps the most important outcome measure for social interaction, is only partially affected by resonance, as is illustrated by the poor correlation (0.48, Figure 2). Normalized resonance may not lead to improved understandability if articulation does not improve[18]. Compensatory articulation is common among patients with 22q11.2DS.

Prognostic factors

Nearly half of our patients had residual hypernasality following velopharyngoplasty. Given the costs and potential complications associated with this procedure, can we identify this subset before subjecting them to surgery? As suggested by Witt et al.[62], suboptimal postoperative functional outcome may represent errors in patient selection rather than errors in operative technique. At our center, all children with 22q11.2DS and residual speech problems following intensive speech therapy undergo velopharyngoplasty. Preoperative prognostic factors have been sought to determine whether it can be predicted which patients are less likely to benefit from surgery. Studies in the larger VPD population including those with 22q11.2DS and non-syndromic cleft palate present conflicting results regarding the predictive value of the preoperative factors we tested.

Like Losken et al. [15], who are the only previous group to report on prognostic factors for postoperative outcome in patients with 22q11.2DS, we did not find gender to be a predictive factor. In the larger VPD population including non-syndromic patients, Kasten et al.[33] found that males had worse postoperative speech scores than females, Sie et al.[11] found that females had worse scores, while four larger studies showed that gender was not a predictor for outcome[11, 14, 19, 20, 63].

One may postulate that those undergoing surgery at an older age may be disadvantaged since compensations are more ingrained and their brains have less plasticity to relearn speaking techniques. However, neither we nor Losken et al.[15] found age to be an outcome predictor. In the larger VPD population including non-syndromic patients, some studies found that an older age at surgery led to worse postoperative results[18, 36, 40, 63, 64], while others found that older patients did not have a poorer outcome [14, 65, 66, 67, 68] but in fact had a better outcome[33]. It is impossible to draw a general conclusion because these studies use different methods: some studies compare the mean ages of patients with successful outcome to those without, while others, like ours, test the success rate above and below a below a cut off age.

The murine model for 22q11.2DS has hypoplastic branchiomeric muscles[69, 70] and aberrant cranial nerves [71]. The clinically hypodynamic pharynx in patients with 22q11.2DS [21, 22, 56] echoes a neuromuscular component in the etiology of VPD. We expected to find that patients without good lateral pharyngeal wall adduction would be less likely to attain normal resonance following a (modified) Honig velopharyngoplasty, but failed to find a significant relationship. This may be explained by the presence of a pharyngeal flap changing postoperative lateral pharyngeal wall adduction[72]. In the larger VPD population including non-syndromic patients, Sie et al.[10] did not find worse postoperative outcomes among patients with less lateral wall movement, but larger studies did[35-38]. Likewise, in our study, patients with poor or moderate velar elevation were not more likely to remain hypernasal than their counterparts with good velar elevation. Witt et al.[62] also did not find a correlation between velar activity and postoperative speech outcome in a VPD population including non-syndromic patients.

We did not find preoperative nasalance or understandability to be predictive for residual hypernasality after surgery. In the larger VPD population including non-syndromic patients, preoperative speech scores have been predictive for postoperative speech scores [14, 36, 63]. In patients with 22q11.2DS, Losken et al.[15] found that lower preoperative nasalance scores correlated with a decreased need for surgical revision.

In our study population, patients with a palatal defect were not more likely to remain hypernasal. Likewise, those who underwent intravelar veloplasty prior to pharyngeal flap surgery did not fare worse than those who did not. This may affirm the adequacy of the (modified) Honig velopharyngoplasty technique for correcting anatomical aberrations. In the larger VPD population including non-syndromic patients, only de Buys Roessingh et al.[39] found that patients with palatal defects who underwent velopharyngoplasty had worse postoperative speech outcomes, while all other studies found no predictive value[11, 36, 63, 67].

While adenoidectomy predisposes to VPD[24,73,74], in our study prior adenoidectomy was not predictive for outcome. Likewise, Witt et al.[75] found no overt correlation between removal of lymphoid tissue and outcome in the larger VPD population including non-syndromic patients.

Since the adenoids are often hypoplastic in 22q11.2DS[76,77] there may not be a real difference between the groups who did and did not undergo adenoidectomy.

Hearing loss in 22q11.2DS can be sensorineuronal[78] or conductive following recurrent otitis media[47]. As postulated by Willging[79], hearing loss hampers VPD resolution since it reduces the patient's ability to self-correct the problem. Albery et al.[65] did not find hearing to be prognostic in the larger VPD population including non-syndromic patients and we did not find a correlation between preoperative hearing loss and postoperative resolution of hypernasal resonance in patients with 22q11.2DS.

Those with a higher IQ may more readily learn to employ the new anatomical situation after surgery for understandable speech. In the larger VPD population including non-syndromic patients, Moll et al.[40] found that IQ is prognostic for postoperative speech outcome while Albery et al.[65] did not find intelligence to be prognostic. We did not find IQ to be prognostic in our 22q11.2DS population. All our patients had speech therapy before and after surgery, however the amount was not documented in the charts and therefore unavailable for testing as a prognostic factor. Speech therapy is essential in learning to speak understandably [80,81].

Major weaknesses of this study include: that speech had not been evaluated by speech therapists who were blinded to the study, that previous live assessments by speech therapists preclude controlling the data, and that much data is missing. Admittedly, being able to review recordings and having more complete data would be preferred, but these ideals are impossible to realize in a retrospective study. Yet, a retrospective design was necessary to yield the largest number of patients.

Although our center is a referral center, and we inventoried data from the past 20 years, we did not have a large enough population to definitively refute the null-hypothesis. Performing a sample size calculation with our ratio of patients with poor outcome to those who attained normal resonance (1:1), to find a prognostic factor that is deemed clinically significant when 70%

of the patients with poor outcome have the factor while only 40% of patients with normalized resonance do, using an alpha of 0.05, a power of 0.8, and a continuity correction, yields a necessary sample size of 96 patients (48 per group). With those assumptions, even with complete data for all the 22q11.2DS patients treated at our center in the past 20 years we would not have sufficient patients. A multicenter cohort study will therefore be necessary to get sufficient numbers to find prognostic factors for postoperative resonance in 22q11.2DS and inform parents whether their child is expected to benefit from surgery.

Conclusion

In patients with 22q11.2DS and VPD, this chart review shows that residual hypernasality persisted in many patients after primary velopharyngoplasty. Resonance continued to change years after surgery, making conclusions about postoperative outcome questionable. No preoperative prognostic factors were found for residual hypernasality and/or undergoing revision surgery. A prospective study or a meta-analysis of current data from multiple centers is needed to elucidate prognostic factors.

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CHAPTER 6

Surgical management of velopharyngeal dysfunction in patients with 22q11.2 deletion syndrome

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Abstract

Background: Speech outcomes after surgical management of velopharyngeal dysfunction (VPD) in children with 22q11.2 deletion syndrome (22q11.2DS) have been reported to be significantly poorer than those observed in non-syndromic children. Results may be optimized by carefully tailoring surgical management to each child's specific anatomy and function. Here, the experiences of pharyngoplasty carried out by a single surgeon over a 10-year period are reviewed.

Methods: The authors performed a retrospective chart review of all patients who underwent posterior pharyngeal flap surgery and sphincter pharyngoplasty by a single surgeon between 1998 and 2007. Deletion status was confirmed by FISH or MLPA. Perceptual speech evaluations were carried out pre- and postoperatively using the "Pittsburgh Weighted Values for Speech Symptoms Associated with VPI". Complication rates and the need for pharyngoplasty revision were recorded.

Results: 62 patients (26 male; 36 female) underwent pharyngoplasty, 40 of whom (17 male; 23 female) were diagnosed with 22q11.2DS. The average age at the time of surgery was 7.5 years in patients with 22q11.2DS and 6.8 years in patients without 22q11.2DS. Both groups showed improvement in velopharyngeal function after surgery. Seven patients (4 with 22q11.2DS; 3 without) required revision due to persistent VPD. Two patients (1 with 22q11.2DS; 1 without) required revision due to symptoms of obstructive sleep apnea.

Conclusion: Posterior pharyngeal flap surgery and sphincter pharyngoplasty are effective treatments of VPD in patients with and without 22q11.2DS. In contrast to prior reports, the need for revision was not found to be higher in patients with 22q11.2DS.

Introduction

22q11.2 deletion syndrome (22q11.2DS) is the most common genetic cause of VPD. Over one third of cases of isolated VPD have been reported to be associated with the deletion[1]. 22q11.2DS is a microdeletion syndrome with a prevalence of approximately 1 in 4000 live births[2, 3, 4]. Its phenotype is highly variable including multiple anomalies such as cardiac malformations, immune deficiencies, facial dysmorphia, and developmental difficulties[5, 6]. VPD is prevalent in 22q11.2DS and may be of complex etiology. Factors that may contribute to VPD in patients with 22q11.2DS include cleft palate, submucosal cleft palate, adenoid hypoplasia[7], velopharyngeal hypotonia[8], and velopharyngeal disproportion[9]. Ruotolo et al. have recently reported that several anatomic factors, including platybasia, contribute significantly to velopharyngeal disproportion in affected patients [9].

Speech outcomes after surgical management of velopharyngeal dysfunction in children with 22q11.2DS have historically been reported to be significantly poorer than those observed in non-syndromic children[10, 11, 12]. It is likely that the differences in velopharyngeal function and anatomy noted above contribute to the relatively high rate of surgical revision in 22q11.2DS. Tailoring surgical technique to pre-operative velopharyngeal anatomy and function may improve the outcome of pharyngoplasty. By employing this principle in the surgical management of 22q11.2DS patients with VPD, speech outcome need not be poorer than that of patients without 22q11.2DS. Here, the results of surgical management of VPD in 22q11.2DS are reported and compared to those observed in non-syndromic patients with VPD treated over a decade by a single surgeon.

Methods

Demographics

A retrospective chart review of all patients who underwent surgical management for VPD by the senior author (REK) between 1998 and 2007 was performed. Prior to surgery, all patients were screened by a geneticist, and those with any clinical features of 22q11.2DS were tested for the 22q11.2 deletion using fluorescence in situ hybridization (FISH) or multiplex ligation-dependent probe amplification (MLPA). Patients who had previously undergone pharyngoplasty, patients with other syndromes, and those with a follow up of less than 12 months were excluded from this analysis.

Pre-operative assessments

All patients were evaluated by a multidisciplinary cleft palate team. A single pediatric plastic surgeon (REK) carried out intraoral examination of all patients, noting the presence of cleft palate, submucosal cleft palate, or bifid uvula. The presence and size of the tonsils was recorded. When tonsillar hyperplasia was observed, tonsillectomy was performed prior to pharyngoplasty in order to reduce the risk of post-operative obstructive sleep apnea (OSA). All patients with 22q11.2DS underwent a cervical MRI to determine the anatomic course of the carotid arteries. Perceptual speech assessment was made by a single speech pathologist (CBS) and scored using the Pittsburgh Weighted Values for Speech Symptoms Associated with VPI (velopharyngeal insufficiency). Resonance was recorded as severely hypernasal, moderately hypernasal, mildly hypernasal, normal, or hyponasal. Nasal air emission was classified as severe (audible air escape), moderate (consistently visible on mirror examination), mild (inconsistently visible on mirror examination), and normal (no air emission). Facial grimace and the the pitch and quality of the voice were assessed, and articulation errors were noted. These factors together were combined into a total speech score defined by the Pittsburgh Weighted Values (0 = competent velopharyngeal mechanism; 1-2 = competent to borderline competent; 3-6 = borderline to borderline incompetent; 7 or greater = incompetent).

Imaging and surgical technique

When perceptual speech evaluation indicated the presence of VPD, patients underwent imaging of the velopharynx using nasendoscopy or multi-view videofluoroscopy to evaluate velopharyngeal anatomy and function. The degree of velar elevation and lateral pharyngeal wall movement were rated as normal, decreased, or absent. Velopharyngeal closure pattern was noted, as was the attempted level of closure. When the closure pattern was coronal in nature and the gap was small (< 0.5 cm.), a sphincter pharyngoplasty was performed, with care taken to tailor the level of inset on the posterior pharyngeal wall to the site of attempted velopharyngeal closure. All other patients underwent creation of a superiorly-based posterior pharyngeal flap with its width tailored to the velopharyngeal gap size and lateral pharyngeal wall motion, as described by Argamaso[13]. In all cases, the flap was based at the level of attempted velopharyngeal closure as determined by pre-operative imaging.

Post-operative assessments

After pharyngoplasty, all patients were followed for a minimum of 12 months. Post-operative speech assessment was performed using the Pittsburgh Weighted Values. In addition, all patients were clinically evaluated for upper airway obstruction. Presence of snoring and/or symptoms of OSA were noted. When signs or symptoms of OSA were noted, patients were further evaluated using polysomnography. The need for revisional surgery, either for persistent VPD or for obstructive sleep apnea, was recorded, as were speech outcomes after revision. Other complications, such as bleeding, infection, or dehiscence were also documented.

Statistical analysis

For the Pittsburgh Speech Scores, a student t-test was used. All other values were analyzed for in-group and between-group differences using Fisher exact test. Only complete result-couples were used. Statistical significance was defined as $P < 0.05$.

Results

Between 1998-2007, 90 patients (34 male; 54 female) underwent pharyngoplasty for the management of VPD by the senior author. Excluding patients with genetic syndromes other than 22q11.2DS, those with a follow-up of less than 12 months, and those who had undergone previous pharyngoplasty, 62 patients (36 female; 26 male) met the criteria for analysis. Of these, 40 (23 female; 17 male) had a confirmed 22q11.2 deletion. The average age at the time of surgery was 7.5 years (range 3.9 - 16.3 years). The remaining 22 patients (13 female; 9 male) did not have a 22q11.2 deletion and had an average age at surgery of 6.8 years (range 3.9 - 16.6 years) (Table 1).

Of the 40 patients with 22q11.2DS, two had previously repaired cleft palate, four had unrepaired submucosal cleft palate, three had unrepaired occult submucosal cleft palate diagnosed by nasendoscopy, and four had isolated bifid uvula. Thus, 32.5% of 22q11.2DS patients had anatomic palatal abnormalities. All 22q11.2DS patients with VPD associated with submucosal clefts underwent pharyngoplasty without palatoplasty. Of the 40 22q11.2DS patients with VPD, 33 (82.5%) underwent posterior pharyngeal flap surgery and 7 (17.5%) underwent a sphincter pharyngoplasty. The average length of follow-up in the 22q11.2DS group was 2.4 years (range 1 - 6.3 years). Of the 22 patients without 22q11.2DS, five had previously repaired cleft palate, and three had a previously repaired submucosal cleft palate Thus, 36.4% of non-deleted patients had anatomic palatal abnormalities. All 22 patients in the non-22q11.2DS group underwent posterior pharyngeal flap surgery. The average length of follow-up in this group was 2.0 years (range 1 - 6.4 years) (Table 1).

	22q11.2 DS group (n=40)	Non-22q11.2 DS group (n=22)
Female	23 (57.5%)	13 (59.0%)
Male	17 (42.5%)	9 (40.9%)
Cleft palate	2 (5%)	5 (22.7%)
Submucosal cleft palate	4 (10%)	3 (13.6%)
Occult submucosal cleft palate	3 (7.5%)	0 (-)
Bifid uvula	4 (10%)	0 (-)
Mean age at surgery (range)	7.5 years (3.9 - 16.3 years)	6.8 years (3.9 - 16.6 years)
Mean follow up (range)	2.4 years (1 - 6.3 years)	2.0 years (1 - 6.4 years)
Posterior pharyngeal flap	33 (82.5%)	22 (100%)
Sphincter pharyngoplasty	7 (17.5%)	0 (-)

Table 1. Characteristics of the 22q11.2DS and non-22q11.2DS groups.

Pre-operative imaging

All patients with 22q11.2DS underwent cervical MRI to evaluate the carotid arteries prior to surgery. Nine of 40 (22.5%) demonstrated medial deviation of the internal carotid artery at the level of the velopharynx. Although these findings were used to provide parents with full informed consent, they did not alter the decision to proceed with surgery nor the surgical technique.

Over half of the patients in both groups demonstrated normal velar motion on pre-operative imaging studies (Figure 1). Interestingly, there was no significant difference in velar motion between the two groups. A significant inter-group difference was found, however, in the degree of lateral pharyngeal wall motion. Of the 22q11.2DS patients, 42.5% were noted to have absent lateral pharyngeal wall motion, whereas only 4.5% of non-deleted patients demonstrated absent lateral pharyngeal wall motion ($p < 0.01$) (Figure 2).

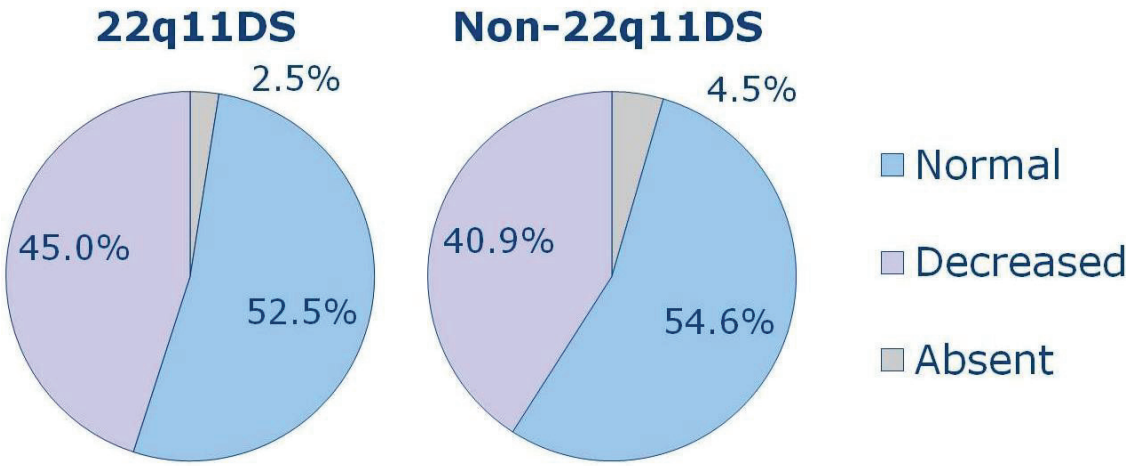

Figure 1A and B. Velar motion pre-operatively as seen with nasendoscopy or multi-view videofluoroscopy. A (left): 22q11.2DS group. B (right): non-22q11.2DS group.

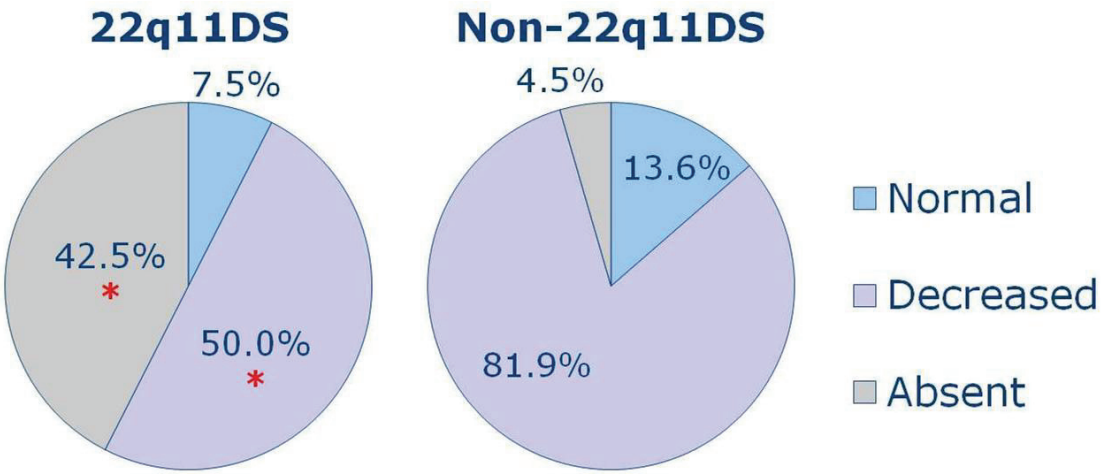

Figure 2A and B. Lateral pharyngeal wall motion pre-operatively as seen with nasendoscopy or multi-view videofluoroscopy. A (left): 22q11.2DS group. B (right): non-22q11.2DS group. * = significant ($p < 0.05$).

Perceptual speech assessment

Patients in both groups demonstrated a significant improvement in velopharyngeal function after surgery ($p < 0.001$ in both groups). Pre-operative assessment revealed a trend toward more severe VPD in the 22q11.2DS group, as compared to the non-22q11.2DS group that approached significance ($p = 0.07$) (Figure 3). After pharyngoplasty, 70% of patients with 22q11.2DS and 50% of patients without the deletion demonstrated normal resonance. Four patients without the deletion (18.2%) were hyponasal, whereas no hyponasality was found in the patients with 22q11.2DS This difference between groups reached significance ($p = 0.02$). Thus, hypernasality was eliminated in a majority of patients in both groups.

Of the 21 patients with 22q11.2DS that showed severe hypernasality pre-operatively, post-operative assessment revealed moderate hypernasality in 3 patients (14.3%), mild hypernasality in 7 patients (33.3%), and normal resonance in 11 patients (52.4%). Of the 16 22q11.2DS patients who demonstrated a moderate degree of hypernasality pre-operatively, one (6.3%) remained moderately hypernasal, one (6.3%) became mildly hypernasal, and 14 (87.5%) demonstrated normal resonance after surgical intervention. All 3 22q11.2DS patients who were mildly hypernasal before surgery had normal resonance after surgery.

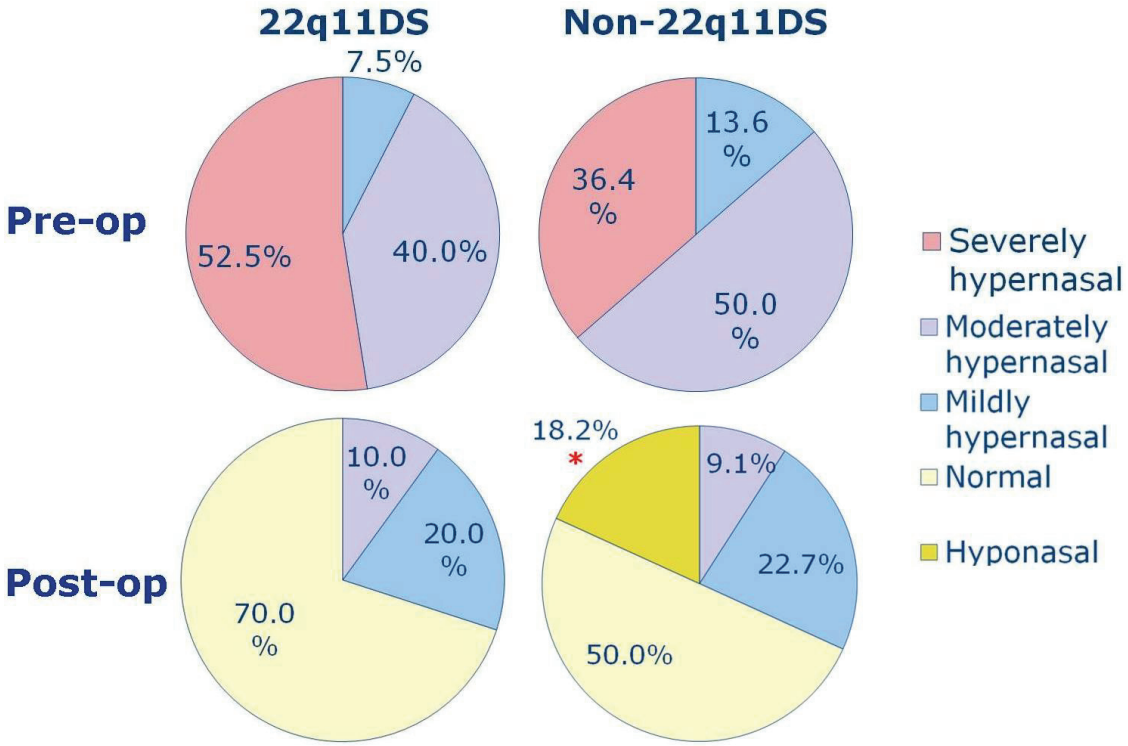

Figure 3A, B, C and D. Resonance. A (top, left): 22q11.2DS group pre-operatively. B (top, right): non-22q11.2DS group pre-operatively. C (bottom, left): 22q11.2DS group post-operatively. D (bottom, right): non-22q11.2DS group post-operatively. * = significant ($p < 0.05$).

A greater percentage of 22q11.2DS patients (52.5%; 21 of 40 patients) demonstrated severe nasal air emission pre-operatively when compared to non-deleted patients (36.4%; 8 of 22 patients) of non-deleted patients, a difference that again approached statistical significance ($p = 0.07$) (Figure 4). Postoperatively, the majority of patients in both groups demonstrated no or mild nasal air emission (80% in 22q11.2DS group and 77.3% in non-22q11.2DS group). The remaining patients in both groups demonstrated moderate nasal air emission after surgery.

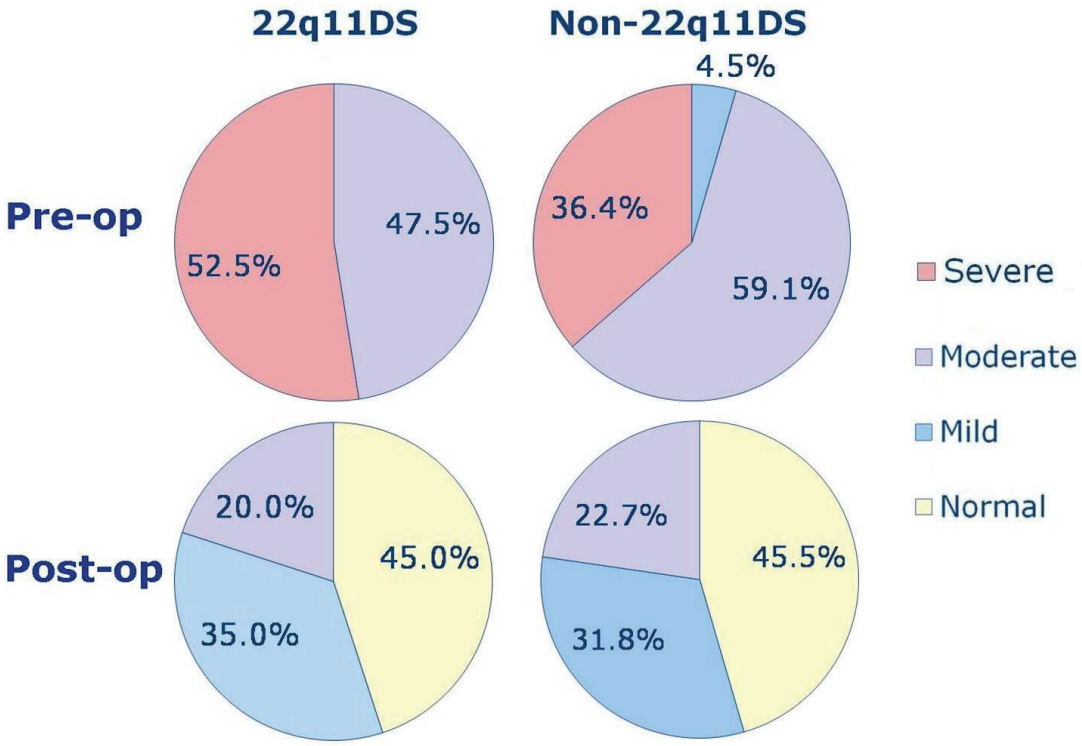

Figure 4A, B, C and D. Nasal air emission. A (top, left): 22q11.2DS group pre-operatively. B (top, right): non-22q11.2DS group pre-operatively. C (bottom, left): 22q11.2DS group post-operatively. D (bottom, right): non-22q11.2DS group post-operatively.

Table 2 notes the prevalence of compensatory articulation errors in both groups. Although the percentage of patients with compensatory misarticulation in the 22q11.2DS group was higher pre-operatively, this did not reach statistical significance. Post-operatively, however, it was observed that the articulation errors persisted in a larger percentage of the 22q11.2DS patients ($p = 0.04$).

	22q11.2 DS group (n=40)	Non-22q11.2 DS group (n=22)	P value
Pre-operative CAE	29 (72.5%)	13 (59.1%)	P = 0.12
Post-operative CAE	17 (42.5%)	4 (18.2%)	P = 0.04*

Table 2. Presence of compensatory articulation errors (CAE) in both groups.
* = significant ($p < 0.05$).

The persistence of compensatory articulation errors is reflected in the total speech scores of the Pittsburgh Weighted Values (Table 3). Complete scores could not be calculated for 11 patients with 22q11.2 deletion syndrome who had missing articulation scores because of poor cooperation. Hence, analysis was only carried out on complete datasets (29 patients out of 40). Both groups improved significantly after pharyngoplasty ($p < 0.001$ for both groups). However, a significant difference was found between the post-operative total scores of the 22q11.2DS group and those of the non-syndromic group ($p = 0.02$). This difference between groups, however, was due entirely to the persistence of articulation errors in the 22q11.2DS group and not to persistent VPD.

	22q11.2 DS group (n=29)	Non-22q11.2 DS group (n=22)	P value
Mean pre-op speech score (range)	12.4 (4-20)	10.3 (4-17)	P = 0.11
Mean post-op speech score (range)	4.3 (0-17)	2.1 (0-5)	P = 0.02*

Table 3. Pittsburgh Weighted Values for Speech Symptoms associated with Velopharyngeal Insufficiency for both groups. * = significant ($p < 0.05$).

Complications

None of the patients suffered complications of post-operative bleeding, infection, or dehiscence. The prevalence of post-operative snoring and of clinically evident signs of obstructive sleep apnea were similar between groups. One patient with 22q11.2DS and one patient without 22q11.2DS required revision of the pharyngoplasty to manage OSA. A second patient with OSA in the non-22q11.2DS group was managed with a continuous positive airway pressure (CPAP) mask.

Need for revision

Of the 40 patients with the 22q11.2 deletion, four patients (10%) required revision of their pharyngoplasty. Three of these patients (7.5%) required revision to manage persistent VPD and one patient (2.5%) required revision to manage symptoms of obstructive sleep apnea. Of the 22 patients without the deletion, three (13.6%) required revision, two for persistent VPD (9.1%) and one for symptoms of obstructive sleep apnea (4.5%) (Tables 4 and 5). Table 4 shows speech improvement after revision in all patients. Table 5 demonstrates that OSA resolved in both patients and resonance remained normal after surgical revision.

	22q11.2 DS group (n=3)			Non-22q11.2 DS group (n=2)		
	Before 1st surgery	After 1st surgery	After 2nd surgery	Before 1st surgery	After 1st surgery	After 2nd surgery
Mean speech score	14.5	11.3	4	8	4	3
Resonance	2 severe 1 moderate	3 moderate	1 mild 2 normal	1 severe 1 moderate	1 moderate 1 mild	1 mild 1 normal
Length of follow-up	-	1.9 yrs	2.1 yrs	-	1.8 yrs	2.6 yrs

Table 4. Revision for persistent VPD in both groups.

	22q11.2 DS group (n=1)			Non-22q11.2 DS group (n=1)		
	Before 1st surgery	After 1st surgery	After 2nd surgery	Before 1st surgery	After 1st surgery	After 2nd surgery
Mean speech score	No report	0	0	10	0	0
Resonance	1 severe	1 normal	1 normal	1 moderate	1 normal	1 normal
Obstructive sleep apnea	-	yes	no	-	yes	no
Length of follow-up	-	1.5 yrs	1.0 yrs	-	1.3 yrs	6.4 yrs

Table 5. Revision for obstructive sleep apnea in both groups.

Discussion

With proper patient selection and surgical execution, both posterior flap pharyngoplasty and sphincter pharyngoplasty are effective treatments for VPD in patients with and without 22q11.2DS. When the type of surgery (sphincter pharyngoplasty versus posterior pharyngeal flap) and width of the pharyngeal flap are tailored based on pre-operative imaging, normal or near normal resonance is obtained in approximately 90% of patients, regardless of 22q11.2 deletion status. In all cases, care must be taken to place the sphincter or pharyngeal flap sufficiently high on the posterior pharyngeal wall at the site of attempted velopharyngeal closure.

Previous reports have described a significantly higher need for revision for persistent VPD in patients with 22q11.2DS compared to non-syndromic patients[10, 11, 12]. Mehendale et al (2004) describe a group of 16 patients with 22q11.2DS who underwent a Hynes pharyngoplasty. Of these, 3 (18.8%) required further surgery[10]. In the study by Losken et al (2006), 32 22q11.2DS patients underwent a sphincter pharyngoplasty[11]. Post-operatively, 6 patients (18.8%) required revisional surgery for persistent VPD. Widdershoven et al (2008) have described a group of 25 patients with 22q11.2DS treated with a palatal lengthening technique for VPD[12]. Of these, 4 patients (16%) subsequently required a sphincter pharyngoplasty for persistent VPD, whereas none of the control patients required revision. In contrast, however, the need for revision for the correction of persistent VPD in our series was no greater in patients with 22q11.2DS (7.5%) than in non-deleted patients (9.1%).

Pre-operatively, the degree of hypernasality and nasal air emission tended to be more severe in patients with the 22q11.2 deletion. After surgery, however, the resonance of patients in the two groups was comparable. Patients in the 22q11.2DS group showed a higher prevalence of persistent compensatory articulation errors and therefore poorer post-operative scores on the Pittsburgh Weighted Values. This was found to persist well beyond the minimum follow-up period of 12 months.

Patients with 22q11.2DS present a complex profile of speech deficits. Most often, speech acquisition is delayed and speech learning proceeds over a significantly longer period of time than in typically developing children. Forty percent of 22q11.2DS children demonstrate features of dysarthria and/or childhood apraxia of speech[14, 15]. This contributes significantly to the severity and longevity of the speech disorders seen in this population. Moreover, the multiple etiological factors in the VPD in 22q11.2DS (palatal disproportion, motor and sensory issues) all contribute to the VPD and must be addressed. In addition, the compensatory errors which develop as a result of the structural palatal deficits in 22q11.2DS may be more difficult to remediate not only because of motor learning difficulties, but also because of cognitive and language learning challenges typically found in children with 22q11.2DS.

It has been reported that VPD in 22q11.2DS may result from velopharyngeal hypotonia and velopharyngeal disproportion. Ruotolo et al[9] found that the platybasia prevalent in 22q11.2DS patients results in an increased velopharyngeal depth and hence an increased velopharyngeal depth/palatal length ratio compared to controls. In addition, patients with 22q11.2DS were found to have significantly greater velopharyngeal width. In the present study, over 40% of patients with 22q11.2DS had no lateral pharyngeal motion as visualized by nasendoscopy and multi-view videofluoroscopy. This was significantly different from the patients without the deletion, 4.5% of whom had absent lateral pharyngeal wall motion. Interestingly, the degree of velar motion did not differ between groups, suggesting that the movement of the velum may not be as severely compromised in 22q11.2DS as that of the lateral pharyngeal walls.

In the majority of patients in this series, upper airway function was preserved, despite the need for very wide flaps in some 22q11.2DS patients. In the cases where obstructive sleep apnea was clinically suspected, polysomnography was performed. Two patients required surgical revision for symptoms of obstructive sleep apnea. One of the limitations of this study is that only those patients with clinical suspicion underwent polysomnography; it is possible that subclinical obstructive sleep apnea in some patients remained undetected. This is illustrated by a study by Saint Raymond et al which examined polysomnographic outcomes before and after pharyngoplasty in 17 patients[16]. They found that the surgical procedure did not alter the apnea-hypopnea index or nocturnal oxygen saturation. However, postoperatively, there was a

reduction in slow wave sleep and an increase in cortical microarousals suggesting an increased respiratory effort sufficient to induce sleep disturbance without evident OSA[16]. Thankfully, upper airway obstruction generally resolves in the first post-operative days. It seems that there may be an inverse relationship between postoperative airway obstruction and residual VPD suggesting that velopharyngeal competence may occasionally come at the cost of, usually temporary, upper airway obstruction.

In conclusion, our results demonstrate that, when individualizing the type and technique of pharyngoplasty to pre-operative assessment of velopharyngeal structure and function, acceptable resonance may be achieved in nearly all patients with and without 22q11.2DS. Our revision rates for persistent VPD are lower than those reported by others, particularly in 22q11.2DS patients, and there was no statistical difference between deleted and non-deleted patients despite a tendency for more severe pre-operative VPD in patients with 22q11.2DS. Total speech scores remained poorer in the 22q11.2DS group, not as a result of persistent VPD but rather as a result of persistent compensatory articulation patterns, underscoring the need for long-term intensive speech therapy in 22q11.2DS patients.

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CONCLUSION

Conclusion

In the **first chapter** of this thesis, the role of a number of anatomical and functional disparities such as cleft palate, adenoid hypoplasia, upper airway asymmetry, platybasia, and muscle hypotonia in the speech problems found in patients with 22q11.2 DS are discussed. So far, the various phenotypical presentations of palatal and speech problems in 22q11.2 DS are not yet precisely described. To date it remains unclear whether findings such as platybasia, adenoid hypoplasia, and upper airway asymmetry are found in all patients with the deletion or only in those with speech difficulties.

The different speech phenotypes in 22q11.2 DS can be distinguished using comprehensive speech assessments and imaging studies detailing how the velopharynx functions when speech is produced. When these phenotypes are well documented, associations can be made with the genetic profiles found in 22q11.2 DS. The most important candidate gene in the deleted region is TBX1, as it is found in the pharyngeal arches and is known to cause palatal anomalies in animal models. However, as the syndrome shows such marked phenotypic variability, it is likely that modifier genes on the remaining allele of 22q11.2 or elsewhere in the genome also play a significant role.

Potential contributing factors to the communication problems in children with 22q11.2 DS are anomalies in neuroanatomy and function. **Chapter two** describes the histologic properties of muscle specimens taken from the pharyngeal constrictor muscle during pharyngoplasty in patients with and without 22q11.2 DS. Based on previous studies with cadavers we expected to find myogenic variability between patients with and without 22q11.2 DS. However, the specimens from the two groups did not differ regarding the presence of increased perimysial or endomysial space, fiber grouping by size or type, internalized nuclei, the percentage type I fibers, or the diameters of type I and type II fibers. Therefore, a myogenic component of the etiology of velopharyngeal insufficiency in children with 22q11.2 deletion syndrome could not be confirmed.

In chapter three, the results of a candidate gene association study in 101 patients with 22q11.2DS are presented. Patients from the Children's Hospital of Philadelphia, USA and the Wilhelmina Children's Hospital Utrecht, The Netherlands were stratified based on palatal phenotype (overt cleft, submucosal cleft, bifid uvula). Single nucleotide polymorphisms (SNPs) in 21 candidate genes for cleft palate were analyzed for genotype-phenotype association. The most significant SNPs were found on the FGF10 gene. Though none of the SNPs remained significant after correcting for multiple testing, the testing of additional samples is likely to improve the statistical power. As such, it offers valuable information in the search for the genetic basis of palatal anomalies in 22q11.2 deletion syndrome.

In chapter four, the surgical outcome of twenty-five patients with the 22q11.2 deletion who underwent palatal lengthening to treat velopharyngeal dysfunction at the Wilhelmina Children Hospital in Utrecht, The Netherlands is presented. These results were compared to those of a group of non-syndromic patients with velopharyngeal dysfunction. In the group of patients with the 22q11.2 deletion four patients (16%) required revisional surgery. In the control group none of the patients required revision. In chapter five, a follow up study on the same groups of patients is described, in an attempt to identify pre- and postoperative factors that may predict surgical outcome. Unfortunately, none of the selected possible factors demonstrated sufficient predictive power.

The surgical outcome of pharyngoplasty in patients with 22q11.2 DS in the Children's Hospital of Philadelphia are presented in chapter six. Interestingly, it does not show higher revision rates for patients with the deletion as compared to non-syndromic patients. This study suggests that, when the type of surgery (sphincter pharyngoplasty versus posterior pharyngeal flap) and width of the pharyngeal flap are tailored based on pre-operative imaging, normal or near normal resonance can be obtained in approximately 90% of patients, regardless of 22q11.2 deletion status.

In conclusion, more research needs to be done documenting and investigating the mechanisms behind the speech problems in 22q11.2 DS. This will aid the chances of a favourable outcome

for the individual 22q11.2 DS patient with speech problems. So far it has proven largely impossible to determine preoperative characteristics which may help us to predict the outcome and to tailor surgery for velopharyngeal dysfunction individually for these patients. Being able to do so in the future would be a major step forward. To achieve this, we recommend that, in order to evaluate the results of speech correcting surgery and therapy, all children with the 22q11.2 deletion need to undergo comparable pre- and postoperative assessments using imaging techniques and thorough speech evaluation. This will facilitate meta-analysis of study outcomes and can help identify the optimal surgical technique(s), timing of surgery, and accompanying speech and language therapy. From this information a widely accepted standard protocol for the treatment of VPI and speech problems in 22q11.2 DS can be developed. We may find that the preferred treatment program depends on variations in genetic profile or velopharyngeal phenotype, but it is possible that the level of experience of the surgeon will remain the dominant factor for success. In either case the knowledge gathered from unified, multi-center pre- and postoperative evaluations will help in the management of this complex and variable syndrome.

NEDERLANDSE SAMENVATTING

Nederlandse Samenvatting

Het 22q11.2 deletie syndroom (22q11.2DS) is een autosomaal dominant overervende aandoening met een geschatte prevalentie van 1 op de 4000 pasgeborenen. Het wordt veroorzaakt door een deletie op de lange arm van chromosoom 22 ter plaatse van band 11.2. In ongeveer 90% van de gevallen is er sprake van een *de novo* deletie. Andere namen waaronder het 22q11.2 deletie syndroom bekend staat zijn het velo-cardio-faciaal syndroom (VCFS), Shprintzen syndroom, Cayler syndroom en Di George syndroom. Veel voorkomende symptomen zijn cardiovasculaire afwijkingen, immunologische stoornissen, psychiatrische stoornissen en velopharyngeale insufficiëntie. De inter- en intrafamiliale variabiliteit is groot. Onderzoek naar de deletie wordt gedaan door middel van FISH- (fluorescence in situ hybridization) of MLPA (multiplex ligation-dependent probe amplification) onderzoek.

Ongeveer een derde van de patiënten met de 22q11.2 deletie heeft een structurele afwijking van het gehemelte, variërend van een bifide uvula tot een volledige palatoschisis. Daarnaast vertoont ongeveer tweederde van de patiënten met 22q11.2DS symptomen van velopharyngeale insufficiëntie, zoals een hypernasale spraak. Dientengevolge hebben patiënten vaak een operatie (pharyngoplastiek) nodig om het overmatig ontsnappen van lucht via de neus tegen te gaan. Het doel van een pharyngoplastiek is om de bestaande velopharyngeale opening te verkleinen. Studies in het verleden hebben aangetoond dat de operatieresultaten van pharyngoplastieken bij patiënten met 22q11.2DS minder gunstig zijn dan bij patiënten met velopharyngeale insufficiëntie zonder de deletie.

In **hoofdstuk een** wordt een introductie gegeven over het voorkomen en de symptomatologie van 22q11.2DS. Hierbij wordt met name aandacht besteed aan de velopharyngeale insufficiëntie die frequent bij patiënten met 22q11.2DS gevonden wordt. Een aantal van de hieraan bijdragende factoren wordt besproken. Vervolgens wordt ingegaan op de mogelijke genetische basis van deze anatomische en functionele factoren.

Een van de mogelijke oorzaken van velopharyngeale insufficiëntie in 22q11.2DS patiënten is pharyngeale spierhypotonie. Hoofdstuk twee beschrijft een studie naar de de histologische kenmerken van de pharyngeale constrictoren in patiënten met velopharyngeale insufficiëntie met en zonder 22q11.2DS. Er werden geen verschillen gevonden tussen patiënten met en zonder de deletie.

In hoofdstuk drie worden de resultaten van een genetisch onderzoek naar de mogelijke rol van kandidaatgenen voor schisis in het voorkomen van gehemelteafwijkingen in 22q11.2DS gepresenteerd. Genetisch materiaal van patiënten uit Nederland en de Verenigde Staten werd onderzocht op de aanwezigheid van SNP's (single nucleotide polymorphisms). Hierbij werd een mogelijke relatie gevonden tussen het optreden van gehemelteafwijkingen en bepaalde SNP's in het FGF10-gen (fibroblast growth factor 10).

In hoofdstuk vier worden de operatieresultaten van pharyngoplastieken bij patiënten met en zonder de 22q11.2 deletie die geopereerd zijn in het Wilhelmina Kinderziekenhuis te Utrecht met elkaar vergeleken. Van de vijfentwintig geopereerde patiënten met de deletie hadden er vier een tweede operatie nodig. Geen van de tweeëndertig geopereerde patiënten zonder de deletie had een tweede operatie nodig.

Hoofdstuk vijf beschrijft de lange termijnresultaten van deze patiënten. De gemiddelde duur van de follow-up was zeven jaar. Er werd gezocht naar mogelijke voorspellende factoren voor het operatieresultaat. Er werden geen significante factoren gevonden.

De operatieresultaten van pharyngoplastieken in het Children's Hospital of Philadelphia, USA worden gepresenteerd in hoofdstuk zes. De operatietechniek die in dit ziekenhuis gebruikt wordt is anders dan die in het Wilhelmine Kinderziekenhuis te Utrecht. Ook in deze studie worden de resultaten van patiënten met en zonder 22q11.2DS met elkaar vergeleken. Hierbij

wordt geen significant verschil gevonden in de mate van succes tussen patiënten met en zonder de deletie.

DANKWOORD

Dankwoord

Graag wil ik alle patiënten en hun families bedanken die mee hebben gedaan aan mijn onderzoek, zowel in Utrecht als in Philadelphia. Zonder deze bijdrage was mijn onderzoek niet mogelijk geweest. Daarnaast hebben hun ervaringen en verhalen mij altijd geïnspireerd en gemotiveerd om mijn werk door te zetten.

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On a more personal note, I would of course like to thank my friends and family. Ula, my dearest paranimf, thank you so much for always listening and being there for me, even if we did not see each other that much in real life. The same goes for those other UC girls: Mariah, Deniz, Linda, and Sarah (my besties in Singapore…). Mijn fantastische SUMMA-vrienden, Rick, Hendrik, Nienke en Petra, bedankt voor de jullie ondersteuning en opbeurende woorden wanneer ik het even niet meer zag zitten. En natuurlijk voor de gezellige etentjes, barbecues, uitjes en weekendjes weg als ik wel even een pauze kon gebruiken! Mijn familie, oma's, ooms, tantes, neven, nichtjes: jullie zijn een inspiratie voor me en ik vind het heel fijn dat jullie deze bijzondere mijlpaal met mij kunnen vieren. Helaas zijn er ook een aantal familieleden die dit niet meer kunnen meemaken, Opa, Opa en Oom Balth, ik weet dat jullie trots zouden zijn geweest. En dan, tot slot, Michiel, Marleen, Anne-Lieke, Thomas, Petra, Wouter, Myra, Pap en Mam: zonder jullie liefde en steun zou dit boekje (zowel de binnen- als de buitenkant) niet bestaan!

CURRICULUM VITAE

Curriculum Vitae

Josine Christine Colette Widdershoven werd op 27 juli 1982 geboren te Nijmegen. In 2000 behaalde zij het eindexamen gymnasium aan het Jeanne d'Arc college te Maastricht. Datzelfde jaar begon zij aan een Bachelor of Science op het University College Utrecht. Na haar Bachelordiploma behaalde ze in 2004 een Master of Science in Molecular Medicine aan het Trinity College in Dublin, Ierland. Vervolgens begon ze in 2004 aan SUMMA, de verkorte geneeskundeopleiding van de Universiteit van Utrecht. Tijdens SUMMA deed zij een wetenschappelijke stage bij prof. dr. Beverly Emanuel, in de Division of Human Genetics and Moecular Biology in het Children´s Hospital of Philadelphia, USA. Haar keuze-co-schap volgde zij op de afdeling Keel-, Neus- en Oorheelkunde van het Antoni van Leeuwenhoek Ziekenhuis te Amsterdam. In augustus 2008 startte zij met de opleiding Keel-, Neus- en Oorheelkunde in het Maastricht Universitair Medisch Centrum bij opleider prof. dr. B. Kremer en waarnemend opleider prof. dr. R.J Stokroos. De eerste perifere opleidingsstage werd afgerond in het Elkerliek Ziekenhuis te Helmond, onder begeleiding van dr. P. Schuil. Op 1 juli 2011 zal zij starten met haar tweede perifere opleidingsstage in het Catharina Ziekenhuis te Eindhoven, onder begeleiding van dr. F. Adriaansen.

SUPPLEMENT I

Supplement I

List of genotyped SNPs on 21 candidate genes using the Affymetrix Genome-wide Human SNP Array 6.0.

Gene	RS-number	Chromosome	Physical Position
MTHFR	rs4845882	1	11765754
	rs4846048	1	11768839
	rs1801131	1	11777063
	rs12121543	1	11777258
	rs9651118	1	11784801
	rs17367504	1	11785365
IRF6	rs2235372	1	208027059
	rs742215	1	208027646
	rs17317411	1	208027937
	rs674433	1	208031498
	rs2235375	1	208032210
	rs17015218	1	208034538
	rs2013196	1	208035034
	rs7552506	1	208036525
	rs17015226	1	208036719
	rs7555285	1	208036978
	rs2236908	1	208038263

	rs2236909	1	208038278
	rs6540560	1	208048696
	rs630984	1	208048916
	rs6696825	1	208048995
	rs17015255	1	208049361
MTR	rs10495384	1	235027978
	rs10733117	1	235030490
	rs10733118	1	235030633
	rs2185208	1	235034979
	rs7526063	1	235038621
	rs4659731	1	235079996
	rs7516758	1	235081234
	rs4456082	1	235081249
	rs2275568	1	235082251
	rs2385500	1	235083531
	rs10802568	1	235094708
	rs2275566	1	235115185
	rs2275565	1	235115299
	rs16834510	1	235116052
	rs10158822	1	235116799
	rs10158222	1	235117305
	rs1266164	1	235117574
	rs16834516	1	235118419

	rs12094071	1	235118656
	rs10925261	1	235119809
	rs3768152	1	235120153
	rs3768155	1	235120345
	rs16834521	1	235121192
	rs1252252	1	235122625
	rs10737812	1	235125451
	rs16834527	1	235125821
	rs4659743	1	235126010
	rs4659745	1	235126130
	rs2275564	1	235126684
	rs3768159	1	235129036
	rs6679990	1	235131853
	rs16834539	1	235132861
TGFA	rs11466307	2	70527498
	rs10496180	2	70527562
	rs11466306	2	70527660
	rs11466304	2	70527741
	rs3771527	2	70528090
	rs7606793	2	70537572
	rs930655	2	70537959
	rs1880039	2	70540560
	rs6729950	2	70543642

	rs3771516	2	70548391
	rs7578860	2	70548697
	rs17005672	2	70561704
	rs17005682	2	70565115
	rs7561997	2	70569556
	rs2902345	2	70570107
	rs17005706	2	70570535
	rs3821262	2	70574514
	rs11466229	2	70576271
	rs4852620	2	70576454
	rs3849385	2	70576556
	rs3771496	2	70577053
	rs3771492	2	70582385
	rs3821261	2	70582761
	rs3755377	2	70586360
	rs404420	2	70587034
	rs7582571	2	70587296
	rs13401501	2	70594757
	rs13401694	2	70594893
	rs11466220	2	70595602
	rs10198315	2	70595681
	rs414399	2	70596820
	rs2863689	2	70597102

	rs4464299	2	70597409
	rs13415412	2	70604161
	rs17005803	2	70604511
	rs6708768	2	70605703
	rs453870	2	70605851
	rs378322	2	70616413
	rs432203	2	70618196
	rs377122	2	70620533
	rs1523300	2	70622873
	rs10489984	2	70625963
	rs11466191	2	70633589
SATB2	rs260761	2	199841435
	rs6735905	2	199855499
	rs4673309	2	199855654
	rs10497832	2	199875181
	rs11891161	2	199875818
	rs1374361	2	199878979
	rs4675475	2	199884288
	rs7569519	2	199888067
	rs7596078	2	199888088
	rs930616	2	199894160
	rs13002186	2	199897725
	rs17197938	2	199908430

	rs3828186	2	199922518
	rs7575191	2	199930423
	rs10497834	2	199934212
	rs16831370	2	199939292
	rs13406992	2	199944386
	rs1348812	2	199944670
	rs1348813	2	199953490
	rs10497836	2	199996963
	rs895882	2	200011260
	rs13392032	2	200017738
	rs13422914	2	200024373
	rs6736601	2	200028263
SUMO1	rs10931992	2	202774664
	rs10185956	2	202790487
	rs10931993	2	202815985
CHRD	rs13084750	3	185577418
FGFR3	rs3860717	4	1761816
	rs746779	4	1763081
MSX1	rs3775261	4	4914646
	rs1042484	4	4915282
	rs4464513	4	4918223
FGF2	rs308392	4	123965061
	rs308394	4	123966333

	rs308395	4	123966392
	rs366192	4	123969401
	rs308412	4	123971467
	rs308416	4	123988076
	rs308413	4	123989039
	rs308434	4	123991278
	rs308435	4	123991468
	rs11938826	4	123992064
	rs308439	4	123993029
	rs17473132	4	123995981
	rs17407577	4	123998791
	rs17006219	4	124001646
	rs6854081	4	124036157
	rs7683093	4	124037535
	rs1476217	4	124037961
MTRR	rs1893579	5	7917354
	rs8752809	5	7918567
	rs17184211	5	7919106
	rs47208168	5	7919764
	rs1334589	5	7920067
	rs4272909	5	7920971
	rs5280202	5	7921156
	rs12820537	5	7934678

	rs7803651	5	7938961
	rs5610175	5	7951089
	rs10983512	5	7959011
FGF10	rs6451758	5	44341272
	rs10462070	5	44341506
	rs17234541	5	44344655
	rs1839090	5	44349039
	rs17316984	5	44351131
	rs980510	5	44354289
	rs10057630	5	44363621
	rs4866891	5	44364027
	rs987642	5	44367662
	rs983374	5	44370740
	rs12517396	5	44395283
	rs339509	5	44396649
	rs17234079	5	44397961
	rs1482672	5	44398526
	rs339502	5	44399764
	rs11750845	5	44408817
	rs1384449	5	44412817
	rs16901816	5	44417455
	rs2973643	5	44417818
	rs2973649	5	44426918

	rs1482680	5	44427899
	rs7718704	5	44428734
	rs10512852	5	44429326
ESR1	rs867240	6	152170335
	rs3844508	6	152181735
	rs9371557	6	152181902
	rs12523770	6	152183315
	rs6917746	6	152184649
	rs9340788	6	152185082
	rs2234693	6	152205028
	rs3853252	6	152211940
	rs4870057	6	152213591
	rs9340817	6	152216799
	rs1709182	6	152217050
	rs712221	6	152221934
	rs9340831	6	152224306
	rs2431260	6	152234024
	rs1709183	6	152235689
	rs11155819	6	152241052
	rs9322335	6	152241822
	rs9322336	6	152242123
	rs9340838	6	152242202
	rs9340844	6	152243317

	rs11155820	6	152245903
	rs9322341	6	152266429
	rs7772475	6	152268615
	rs9340877	6	152268641
	rs9397453	6	152278572
	rs4870061	6	152279161
	rs4458702	6	152282116
	rs988328	6	152282843
	rs2347868	6	152293261
	rs9371562	6	152302335
	rs4583998	6	152302361
	rs1884051	6	152324972
	rs2982694	6	152327380
	rs9383951	6	152337306
	rs3020327	6	152343338
	rs2144025	6	152349399
	rs12664544	6	152350666
	rs13216134	6	152370177
	rs1569788	6	152370309
	rs9397074	6	152371408
	rs9340955	6	152371894
	rs9340958	6	152372366
	rs2982705	6	152389551

	rs3020422	6	152390451
	rs6941035	6	152420394
	rs9341016	6	152423691
	rs2273206	6	152424004
	rs2273207	6	152424018
	rs2207396	6	152424075
	rs3798571	6	152426493
	rs3778080	6	152426929
	rs3778081	6	152428321
	rs3798573	6	152431055
	rs3020375	6	152431661
	rs9479191	6	152434853
	rs3778089	6	152435454
	rs3822990	6	152447658
	rs750686	6	152449819
	rs9341038	6	152451346
	rs9322359	6	152451715
	rs2228480	6	152461788
	rs3798577	6	152462823
	rs9341074	6	152464148
	rs2813544	6	152467275
	rs1543403	6	152470397
FGFR1	rs2288696	8	38405382

	rs10108561	8	38410369
	rs2978073	8	38414699
	rs11777067	8	38417804
	rs4733946	8	38438506
	rs6996321	8	38441503
	rs12677355	8	38449100
FGFR2	rs10749418	10	123225051
	rs1649163	10	123230277
	rs1649202	10	123230615
	rs3135819	10	123231112
	rs3135810	10	123232770
	rs2278202	10	123233187
	rs1649200	10	123233720
	rs3135807	10	123234667
	rs1613776	10	123234824
	rs2433759	10	123242522
	rs2912753	10	123247392
	rs3135785	10	123249997
	rs3135734	10	123314879
	rs2912787	10	123315528
	rs2981449	10	123315745
	rs3135730	10	123315784
	rs2981432	10	123316636

	rs10736303	10	123324447
	rs3116478	10	123325790
	rs3135715	10	123344716
FGF3	rs948133	11	69332787
	rs12577891	11	69332822
	rs10908228	11	69336099
	rs11263591	11	69338136
	rs3895665	11	69346074
	rs3892955	11	69346602
	rs11263596	11	69346904
PVRL1	rs4459318	11	119046988
	rs10790332	11	119058888
	rs4936492	11	119065659
	rs11217400	11	119065990
	rs11217408	11	119069693
	rs11217409	11	119069706
	rs7950059	11	119070705
	rs1467051	11	119073511
	rs7945395	11	119074947
	rs7945424	11	119075012
	rs10892434	11	119078599
	rs715849	11	119080934
	rs4938717	11	119082121

	rs12421432	11	119109057
	rs12421434	11	119109077
TGFB3	rs4252348	14	75494617
	rs2284791	14	75498789
	rs3917203	14	75499082
	rs3917202	14	75499224
	rs3917201	14	75499308
	rs3917180	14	75505698
	rs3917148	14	75516274
	rs11466414	14	75517603
FGF7	rs16962445	15	47506171
	rs2413944	15	47524043
	rs17478618	15	47524110
	rs16962490	15	47530920
	rs17479003	15	47532712
	rs10519227	15	47533656
	rs7167041	15	47542036
	rs4480740	15	47543134
	rs12148764	15	47543373
CBS	rs8133239	21	43342773
	rs466791	21	43343023
	rs2839631	21	43343155
	rs719038	21	43344013

	rs2124458	21	43348749
	rs2124459	21	43348783
	rs9325622	21	43352770
	rs11203172	21	43353184
	rs4920037	21	43354960
	rs1789953	21	43356005
	rs234706	21	43358419
TBX1	rs9618681	22	18119390
	rs11912973	22	18120562
	rs2238776	22	18137892
	rs4819522	22	18146782

SUPPLEMENT II

Supplement II

Nasometric results

VCFS group

	Standard text			Denasal text		
Pt.nr.	Pre	Post	difference	pre	post	Difference
2	64.9	43.0	21.9			
10	57.8	48.9	8.9	51.3	37.4	13.9
11	68.5	38.9	29.4	60.3	14.0	46.3
13	67.0	58.0	9	67.0	55.9	11.1
17	28.5	45.6	-17.1	12.8	25.8	-13
18				69.9	60.4	9.5
21	65.0	29.6	35.4	85.0	12.3	72.7
22	71.6	40.3	31.3	63.3	25.7	37.7
23	58.0	55.2	2.8	54.0	47.9	6.1
25	51.5	35.0	16.5	48.0	28.0	20.0
Mean	**59.2**	**43.8**	**15.4**	**56.8**	**34.0**	**22.8**

Control Group

	Standard text			Denasal text		
pt.nr.	pre	Post	difference	pre	post	Difference
1	58.0	58.0	0	56.6	56.6	0
2				58.0	21	37
3	52.2	41.8	10.4	39.8	17.7	22.1
4	49.7	41.0	8.7	36.7	22.4	14.3
6	67.8	33.0	34.8	60.6	21.0	39.4
8	61.9	37.0	24.9	53.9	13.1	40.8
9	52.9	46.6	6.3	40.4	20.8	19.6
10	54.6	30	24.6	44.2	30	14.2
11	50.5	30.0	20.5	27.4	22.0	5.4
12	67.6	42.6	20.5	59.0	19.0	40
13				47.5	30.0	17.5
14	36.0	38.3	-2.3	22.3	24.8	-2.5
15	59.0	46.7	12.3	46.0	37.5	8.5
16	52.0	29.0	23	44.0	23.0	21
18	60	21	39			
19	61.0	30.4	30.6	63.0	19.0	46
20	53.6	39.0	14.6	47.1	31.0	16.1
21	52.9	40.5	12.4	47.4	31.8	15.6

22	73.4	43	30.4	53.5	27.0	26.5
23	54	44.5	9.5	48.5	37	11.5
24	37.3	36	1.3	26.8	27.5	-0.7
25	45.0	34.8	10.2			
26	55.8	37	18.8	32.2	13.0	19.2
27	61.4	42.5	18.9	50.0	35.3	14.7
28	50.2	38.5	11.7	39.2	17.0	22.2
29	63.4	47	16.4	56.5	42	14.5
31	53.4	34.6	18.8	42	23	19
32	55.2	32.0	23.2	57.8	24.0	33.8
Mean	55.8	38.3	17.5	46.1	26.3	19.8