GENETIC AND ENVIRONMENTAL FACTORS ASSOCIATED WITH HYPODONTIA

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ABSTRACT

INTRODUCTION

Hypodontia, or tooth agenesis, is the most prevalent craniofacial malformation in humans. It may occur as part of a recognised genetic syndrome or as a non-syndromic isolated trait. Excluding third molars, the reported prevalence of hypodontia ranges from 1.6 to 6.9 %, depending on the population studied. Most affected individuals lack only one or two teeth, with permanent second premolars and upper lateral incisors the most likely to be missing. Both environmental and genetic factors are involved in the aetiology of hypodontia, with the latter playing a more significant role. Hypodontia individuals present a significant clinical challenge for orthodontists because the treatment time is prolonged and the treatment outcome is generally compromised. Hence, the identification of genetic and environmental factors may be particularly useful in the early prediction of this condition and the development of prevention strategies and novel treatments in the future.

OBJECTIVES

The objectives of this study were two-fold: (1) to investigate the association between non-syndromic hypodontia and single nucleotide polymorphisms (SNPs) of candidate genes paired box 9 (PAX9), msh homeobox 1 (MSX1), axis inhibition protein 2 (AXIN2), and ectodysplasin A (EDA); and (2) to examine its association with environmental factors, such as exposure to smoking and alcohol during pregnancy.
MATERIALS AND METHODS

Eighty-nine cases with two specific phenotypes were recruited: (1) individuals with one or more missing permanent lateral incisors; and (2) individuals with one or more missing permanent premolars. These cases were frequency-matched to 253 controls (patients with no missing teeth, excluding the third molars). Self-report data from both the participants and their mothers were collected, while DNA samples (in the form of blood or saliva) were collected from each participant. Both environmental and genetic data were analysed using conventional descriptive methods.

RESULTS

The sample had a mean chronological age of 16.6 years (SD=7.3), with most participants being female (59.6%), and of New Zealand European origin (75.4%). Using multiple logistic regression analyses, it was found that the T-allele of rs12853659 (EDA) was associated with a higher risk of hypodontia (odds ratio, OR = 2.79, P = 0.029), when adjusted for sex and ethnicity; and this was also true for the G-allele of rs2428151 (EDA), which was associated with a higher risk (OR = 2.87, P = 0.043). For PAX9, the A-allele of rs2073242 was associated with a high odds (1.49, 95% CI = 1.01-2.21) of having hypodontia (P = 0.045); however, this attenuated after adjusting for sex and ethnicity. No statistically significant associations were found with the AXIN2 and MSX1 genes. Analysis of the environmental data revealed a significant association between hypodontia and maternal cigarette use during pregnancy (P < 0.01), as well as the number of cigarettes smoked per day (P < 0.05). To determine whether there was a biological gradient with cigarette smoking during pregnancy, maternal cigarette consumption per day was divided into three groups: none, 1 to 9, and 10 or more. A dose-response association was observed (OR = 2.05 (0.73-5.75), P = 0.232; OR = 4.18 (1.49-11.80), P = 0.007, respectively). These findings suggest that greater cigarette smoking during pregnancy resulted in higher odds of having a child with hypodontia. There were no statistically significant differences between the case and control groups in any of the other environmental factors investigated (that is, alcohol and caffeine consumption).
CONCLUSIONS

Hypodontia is a complex condition that is influenced by both genetic and environmental factors. The present study reveals some evidence that polymorphisms of the EDA and PAX9 genes are associated with specific phenotypes of non-syndromic hypodontia. Furthermore, this study is the first to date to test the association between maternal cigarette smoking during pregnancy and having a child with hypodontia. The observed biological gradient strongly suggests an association between tobacco smoking and this dental anomaly. However, larger samples are needed to investigate the association further, as well as to confirm the genetic variants associated with hypodontia.
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DEDICATION

I dedicate this thesis to my wonderful parents
- my late father, Husam Sabar Al-Ani, and my mother, Wasan Ihsan Al-Mukhtar -
and my beautiful grandmother, Balqees Ahmed.

Thank you for all the doors you opened and all the light you shone.
OVERVIEW

The present work, which focuses on the genetic and environmental factors of hypodontia, is divided into eight sections. These sections are organised as follows:

SECTIONS 1 & 2:

GENERAL INTRODUCTION AND LITERATURE REVIEW

A general introduction as well as an overview of the impact of hypodontia is presented in these sections. These sections include a review of the epidemiological, aetiological and clinical features of non-syndromic hypodontia, and an outline of the psychosocial impact of this particular condition is given.

SECTION 3:

CORE METHODS AND MATERIALS

The methodological details of the present work are presented in this third section. Included is a brief outline of the design of the study, as well as details of the data collection procedures and statistical analyses involved.

SECTION 4:

ENVIRONMENTAL FACTORS AND HYPODONTIA

The role of environmental risk factors in the aetiology of hypodontia is reviewed. This section then describes, analyses and discusses the results of the case-control study carried out to investigate the association between non-syndromic hypodontia and environmental factors such as maternal smoking and alcohol consumption.
SECTION 5:

GENETIC FACTORS AND HYPODONTIA

The role of genetic risk factors in the aetiology of hypodontia is reviewed. This section also includes a detailed description of the methods and materials, analysis and discussion of the results from the cases-control carried out to investigate the association between non-syndromic hypodontia and polymorphisms of four specified genes.

SECTION 6:

GENERAL DISCUSSION AND CONCLUSIONS

The sixth and final section of this work includes a general discussion of the study’s design and findings. In particular, a discussion of the strengths and limitations of the study and the future directions for research in this field is given.

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<td>Analysis of variance</td>
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<tr>
<td>AXIN2</td>
<td>Axis inhibition protein 2 gene</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<td>CI</td>
<td>Confidence Intervals</td>
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<td>CL/P</td>
<td>Cleft lip and/or palate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDA</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>Hh</td>
<td>Hedgehog gene</td>
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<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
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<td>MAF</td>
<td>Minor allele frequency</td>
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<td>MSX1</td>
<td>Msh homeobox 1 gene</td>
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<tr>
<td>OHRQoL</td>
<td>Oral health-related quality of life</td>
</tr>
<tr>
<td>OPG</td>
<td>Orthopantomogram radiograph</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>P-value</td>
<td>Calculated probability</td>
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<tr>
<td>PAX9</td>
<td>Paired box 9 gene</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>rs</td>
<td>Reference single nucleotide polymorphism</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
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TNF  Tumour necrosis factor
WNT  Wingless related integration pathway
I. INTRODUCTION

Hypodontia, or agenesis of teeth, is the most common craniofacial malformation in humans (Matalova et al. 2008). It presents heterogeneous phenotypes ranging from a single congenitally missing tooth, to more than 6 teeth (oligodontia), excluding third molars (Wang et al. 2016). It may occur as part of a recognised genetic syndrome or as a non-syndromic isolated trait (Stockton 2008). Hypodontia can have a negative effect on facial aesthetics and quality of life. Indeed, it has been found that children with multiple missing teeth experience substantial functional and psychosocial impairments from the condition (Locker et al. 2010). Furthermore, affected individuals present a significant clinical challenge for orthodontists because the treatment outcome is generally compromised.

The exact mechanisms that lead to teeth agenesis are still largely unknown; however, it is believed that hypodontia may follow an autosomal dominant, or an autosomal recessive pattern of inheritance with considerable variation in both penetrance and expressivity (Ahmad et al. 2006; Alvesalo 1969; Arte 2001; Cobourne 2007; Goldenberg et al. 2000; Pirinen 2001; Vastardis 2000). A multifactorial model has been proposed to explain the variation in the number and size of teeth, where specific thresholds for hypodontia and hyperdontia (supernumerary teeth) are influenced by both genetic and environmental factors (Brook 1984).

The aims of the present study were to: (1) investigate the association between non-syndromic hypodontia and single nucleotide polymorphisms (SNPs) of candidate genes paired box 9 (PAX9), msh homeobox 1 (MSX1), axis inhibition protein 2 (AXIN2), and ectodysplasin A (EDA); and (2) examine the association between non-syndromic hypodontia and environmental factors, such as exposure to smoking and alcohol during pregnancy.

Identification of the polymorphisms in the genes responsible for tooth agenesis and quantifying the exact role of environmental and epigenetic factors in regulating tooth development has important clinical implications. The identification of environmental risk factors (particularly if they can be combined with genetic covariates) may provide the best opportunity for both personalised and population-level prevention. In the future, it is hoped that better knowledge of the
molecular pathways involved in tooth development, along with the rapid advances in stem cell research, will facilitate the exciting prospect of tooth bioengineering. The realisation of regenerative therapies for missing teeth is clearly dependent on knowledge of the genes involved in odontogenesis.
2. LITERATURE REVIEW

2.1 DEFINITIONS & CLASSIFICATIONS

Hypodontia is the most prevalent dentofacial malformation in humans (Matalova et al. 2008). It may occur as part of a recognised genetic syndrome or as a non-syndromic isolated trait (Cobourne and Sharpe 2013; Frazier-Bowers et al. 2002; Stockton et al. 2008). The condition refers to the developmental failure of six or fewer teeth (Nunn et al. 2003). Its phenotypic presentation is varied in terms of severity and, as a result, various terms have been used to describe it. These terms include “congenitally missing teeth”, “tooth agenesis”, “hypodontia”, “oligodontia” and “anodontia”. The term “congenitally missing teeth” is challenging because tooth development is completed after birth, so that the presence or absence of most tooth germs can be verified only during childhood (Nieminen 2009; Nikopensius et al. 2013; Parkin et al. 2009), whereas “congenital” describes a disease or a malformation being present from birth. Tooth agenesis, on the other hand, refers directly to the developmental failure of a tooth. Other terms, such as hypodontia, are more suitable for classifying the type of tooth agenesis present and may be more appropriate in this context (Cobourne 2007). Oligodontia and anodontia are used to describe more severe forms of tooth agenesis, typically the absence of more than six teeth and the entire dentition (Nunn et al. 2003) respectively. Tooth agenesis and hypodontia are the preferred terms in this work, with the latter term limited to missing teeth other than third molars.

2.2 PREVALENCE

2.2.1 DECIDUOUS DENTITION

Tooth agenesis is considered rare in the deciduous dentition and is not as common as in the permanent dentition. A strong association exists between hypodontia in the primary and permanent dentitions, with reports of children with primary teeth hypodontia showing absence of the corresponding successor teeth (Olmsted 2011). In fact, children with hypodontia in the primary dentition nearly always show hypodontia of the successors (Arte 2001; Bailleul-Forestier et al. 2008). A prevalence of less than 1% has been described in Caucasian populations (Nieminen 2009), although it has been reported to be much higher in Japanese
populations (Yonezu et al. 1997). The prevalence of tooth agenesis in New Zealand appears to be consistent with that seen in Europe (Whittington and Durward 1996). The deciduous maxillary lateral and mandibular central incisors account for 50% to 90% of affected deciduous teeth (Nieminen 2009). Most cases present as unilateral hypodontia, with mostly one or two teeth missing (Arte 2001). No significant sex difference in prevalence has been reported from any of the populations studied (Arte 2001).

2.2.2 PERMANENT DENTITION

The prevalence of hypodontia, which may be increasing with time, ranges from 1.6% to 36.5%, depending on the population studied (Matalova et al. 2008). At least 1 in 5 individuals lack a third molar, while most individuals with hypodontia (80%) lack only one or two teeth (Lidral and Reising 2002; Vastardis 2000). A meta-analysis - which included 33 studies from North America, Australia and Europe - investigated the prevalence of non-syndromic tooth agenesis and found a higher prevalence in Europe (5.5%) and Australia (6.3%) than in North America (Polder et al. 2004). Most individuals were missing only one or two permanent teeth, with very few missing more than six. The most commonly missing teeth were the mandibular second premolars and the maxillary lateral incisors (Polder et al. 2004; Symons et al. 1993). Notably, the prevalence of tooth agenesis has reportedly increased during recent decades (Mattheeuws et al. 2004). However, there is no empirical evidence to support whether this apparent increase is due to more advanced screening and diagnosis or other factors.

Hypodontia is typically associated with a number of classical features, including the site of agenesis and the size of the adjacent teeth. No clear difference in tooth agenesis has been found between the maxilla and the mandible (Polder et al. 2004), although there was one early study that found the mandible to be more frequently affected than the maxilla (Wisth et al. 1974). Comparing bilateral and unilateral agenesis, Polder et al. (2004) found that bilateral agenesis of maxillary lateral incisors occurred more often than unilateral agenesis. For the other teeth, such as the second mandibular premolar, unilateral agenesis was more common (Polder et al. 2004). There appears to be no significant sex difference in missing primary teeth (Hobkirk et al. 1994), although in the permanent dentition, most authors report a small albeit non-significant predominance of hypodontia in
females (Davis 1987; Muller et al. 1970). One meta-analysis, however, found a significant sex difference, with the prevalence of hypodontia being 1.4 times higher in females than in males (Polder et al. 2004).

2.3 FEATURES ASSOCIATED WITH HYPODONTIA

Tooth agenesis is often an isolated anomaly, but it can also be associated with oral clefts and several well-defined malformation syndromes (Arte 2001). For example, hypodontia is a common trait in cleft-lip and/or palate (CLP) patients (Satokata and Maas 1994). The prevalence of hypodontia is higher in more severe clefting cases, with the upper lateral incisor being the most frequently affected tooth in the cleft area (in either dentition) (Arte 2001; Nieminen 2009). In these patients, hypodontia in regions outside the cleft field is also more common than in the general population (Ranta 1986). Other conditions that have hypodontia as one of their features include Down’s Syndrome and ectodermal dysplasia. In these syndromes, there is a characteristic pattern of agenesis that is usually different from the overall population (Nieminen 2009). Moreover, recent data suggest that hypodontia shares some common pathways with particular kinds of cancer (Küchler et al. 2013; Lammi et al. 2004).

It is not known whether individuals with hypodontia have characteristic skeletal features and growth patterns, although some evidence suggests that hypodontia patients have significantly different craniofacial features from those with no missing teeth (Hobkirk et al. 2011). What is known is that tooth agenesis, especially in its severe forms, contributes to abnormal occlusion and is often associated with various anomalies in other teeth (Nieminen 2009). These include delays in development, ectopic eruption, reduction in tooth dimensions and morphology, shortened roots, taurodontia, and enamel hypoplasia (Arte 2001).

2.3.1 DENTAL FEATURES

Microdontia is a widely reported feature of hypodontia in case reports and case series (Hobkirk et al. 1994). This condition, which can affect one or more teeth, may be seen in either dentition (Hobkirk et al. 2011; Pinho et al. 2007). In addition, microdontia is genetically determined and presents in its severest form as ectodermal dysplasia (Hobkirk et al. 2011). It is also present in patients who have
had chemotherapy or radiation of the jaws earlier in childhood (Oguz et al. 2004). Brook proposed that microdontia and hypodontia are genetically linked as a continuum of tooth size, where a tooth will fail to develop if the tooth germ does not reach a particular tooth size and tooth number “thresholds” (Brook 1984).

Delays in tooth development are another common feature, whereby the absence of a permanent successor delays the normal resorption of the roots of the primary teeth. In fact, the primary teeth may be retained for long periods of time, and occasionally up to 40 or 50 years (Haselden et al. 2001). Meanwhile, approximately 46% of individuals with tooth agenesis also have short roots of other permanent teeth (Arte 2001). In addition, an association between taurodontism and hypodontia was found in a Dutch study, where taurodontism of the lower first molars was present in 29% of oligodontia patients but only 10% of controls (Schalk-van der Weide et al. 1993).

Another common feature of hypodontia is the ectopic eruption of permanent teeth. This is likely to be caused by both the lack of adjacent teeth available to guide the eruptive process, and the deficiency of space into which they may erupt. Transposition of teeth is also seen more commonly in individuals with hypodontia (Peck 1993; Peck 2002). Tooth agenesis is also associated with enamel hypoplasia, diminutive or peg maxillary lateral incisors, infraocclusion of the primary molars, and palatal displacement of the upper canines (Baccetti 1998; Pirinen et al. 2001). Intra-orally, retroclined and over-erupted lower incisors contribute to a greater overbite (Carter et al. 2003). Generalised spacing and rotations of both adjacent and non-adjacent teeth to missing mandibular second premolars are also commonly seen (Baccetti 1998). Some of these features are evident in Figure 2.1.

2.3.2 SKELETAL FEATURES

In hypodontia patients, there is a tendency towards a lower mandibular plane angle, associated with a smaller lower anterior facial height and lip protrusion (Chung et al. 2000). Other features include smaller maxillary and mandibular lengths and a tendency towards a Class III skeletal relationship (Ogaard and Krogstad 1995). The short face height, along with the large freeway space, which is typical of hypodontia patients, may make them appear over-closed (Hobkirk et al. 2011). It was initially reported that the maxilla was shorter and more retrognathic.
and the upper incisors more proclined in 9-year-old children with tooth agenesis (Wisth et al. 1974). However, these same children were re-examined in another study and the authors concluded that changes in craniofacial structures from 9 to 16 years of age were about the same as in children without missing permanent teeth (Roald et al. 1982).

In general, dentofacial changes appear to be most prominent in individuals with severe hypodontia, and these are related more to dental and functional compensation rather than to a different growth pattern (Hobkirk et al. 2011; Ogaard and Krogstad 1995).

Figure 2.1. A female patient presenting with several common features of hypodontia. Note the agenesis of the maxillary lateral incisors and the second premolars, the retained primary mandibular molars, the generalised spacing and the deep bite.
2.4 AETIOLOGY

Many theories about the aetiology of tooth agenesis have been suggested in the literature, especially before the more recent genetic information. The multiplicity of tooth agenesis theories suggests a multifactorial aetiology that involves both genetic and environmental regulation. The multifactorial nature of hypodontia entails a brief overview of tooth development and its genetic regulation. This will be followed by an outline of the theories surrounding hypodontia, and a more detailed discussion of the specific genetic and environmental factors that have been implicated in this condition.

2.4.1 TOOTH DEVELOPMENT

Tooth development is a complex process which involves reciprocal interactions between the oral epithelium and ectomesenchyme derived from the neural crest. Studies of mouse tooth development show that during the initiation stage, thickening of the epithelium occurs, and this invaginates into the mesenchyme, creating a tooth bud (Cohn 1957). Within the tooth bud, there is a collection of cells known as the primary enamel knot, and these cells manage this process via signalling proteins. The mesenchyme begins to surround the epithelium to produce initially a cap stage, and later a bell stage. These stages can be seen in the histological slides in Figure 2.2. Mesenchymal cells adjacent to the basement membrane differentiate into odontoblasts, which begin to secrete an organic dentine matrix into which hydroxyapatite crystals are deposited (Hobkirk et al. 2011). The epithelial cells adjacent to the dentine differentiate into ameloblasts, which secrete the enamel matrix and control the mineralisation and subsequent maturation of the enamel (Cohn 1957). The formation and morphology of cusps in premolars and molars is controlled by secondary enamel knots, which develop at the sites where the cusps are to form. These produce folding of the developing tooth germ according to the pre-determined crown morphology (Zhang et al. 2009).

Histodifferentiation in the crown region is then continued in the root. During root development, the odontogenic epithelium extends apically to form Hertwig's root sheath, which controls radicular dentine formation. This later degenerates and leads to the development of cementoblasts. The cementoblasts, in turn, deposit
cementum on the root surface (Khan 2007). Cells in the adjacent dental follicle differentiate into fibroblasts and osteoblasts, and these cells contribute to the formation of the periodontal ligament (Fleischmannova et al. 2008).

Figure 2.2. Adapted from Ten Cate’s Oral Histology (7th Edition), 2000. Histological slides showing the histodifferentiation within the enamel organ through the cap, early bell and late bell stages. (A) Dental lamina, (B) outer enamel epithelium, (C) stellate reticulum, (D) inner enamel epithelium, (E) dental follicle, (F) dental papilla.

Tooth development involves a series of genetically controlled sequential molecular interactions (Galluccio et al. 2012; Thesleff and Cohen 2006). Members of the hedgehog (Hh), wingless related integration site (Wnt), fibroblast growth factor (Fgf) and bone morphogenic protein (Bmp) families are involved in the epithelial-mesenchymal signalling interactions underlying tooth development at the molecular level (Fleischmannova et al. 2008; Dassule et al. 2000). Levels of Wnt and ectodysplasin A (EDA) signalling activity appear to be of particular importance (Cobourne 2013). Mice studies have shown that transgenic overexpression of these pathways in the oral epithelium can produce supernumerary teeth (Ahn et al. 2010; Wang et al. 2009c; Liu et al. 2008; Jarvinen et al. 2006; Kangas et al. 2004; Tucker et al. 2004; Mustonen et al. 2003). Meanwhile, reduced signalling of these factors can result in tooth agenesis (Andl et al. 2002; Tucker et al. 2000; Pispa et al. 1999). Similarly in humans, it has been shown that disrupted EDA signalling or Wnt10A function can cause both syndromic and non-syndromic tooth agenesis (Kantaputra and Sripathomsawat 2011; Headon and Overbeek 1999; Kere et al. 1996). Figure 2.3 summarises the molecules involved in tooth development in humans and mice. Ultimately, disruption of any one of these signalling pathways can affect tooth development and may contribute to developmental dental disorders such as hypodontia.
2.4.2 TOOTH AGENESIS THEORIES

Many theories have been developed to explain tooth agenesis, and most have focused on either genetic or environmental factors, although the importance of both components in the agenesis of teeth is now well recognised. These theories can be considered as either evolutionary or anatomical (Galluccio et al. 2012).

Most of the early studies focused on the evolutional viewpoint, which attributed tooth agenesis to shortening of the maxillo-mandibular complex and the subsequent adaptive reduction of the number of teeth because of the smaller arches. For instance, in 1945, Dahlberg applied Butler’s Field Theory for the evolutionary development of mammalian teeth into the human dentition in order to explain different patterns of agenesis. Four morphological fields (incisors, canines, premolars and molars) were described in each jaw. It was suggested that the more mesial tooth in each field was the most genetically stable and consequently was rarely missing (Hobkirk et al. 2011), while the teeth at the end of each field were less genetically stable. A later theory hypothesised that the last of each “class” were just vestigial bodies that became obsolete during the evolution process (Clayton 1956). Most currently, there is a theory that evolutionary change is working to reduce the human dentition by the loss of an incisor, premolar and molar in each quadrant. According to Vastardis (2000), as humans evolve, the size of the jaws and the numbers of teeth appear to be decreasing (Vastardis 2000).
Other theories focused on an anatomical principle, based on the hypothesis that specific areas of the dental lamina are more sensitive to environmental influences during the maturation of teeth (Galluccio et al. 2012). In support of this hypothesis, Svinhufvud et al. (1988) related the agenesis of the maxillary lateral incisors, the second mandibular premolars and the central mandibular incisors to the fact that they develop in areas of initial fusion of the jaw (Svinhufvud et al. 1988). For example, maxillary lateral incisors develop in the area of the fusion between the lateral maxillae processes and the medial nasal bone process, while the mandibular second premolars originate in another fragile area of the dental laminae (Svinhufvud 1988). Instead, Kjaer and co-workers (1994) argued that the most sensitive area is the one where the innervation develops last (Kjaer et al. 1994).

The proposed effects of both polygenetic and environmental factors on hypodontia represented a paradigm shift in thinking with respect to the aetiology of tooth agenesis. Grahen was the first to consider agenesis as a hereditary anomaly whose transmission is determined by a dominant autosome, with incomplete penetrance and variable expressivity (Grahen 1956). Later, Brook’s theory claimed a significant association between hypodontia and microdontia, with sex differences in tooth size and number (Brook 1984). According to Brook, each anomaly occurred more frequently in first-degree relatives than the population sample, and this suggested that, the more severe the hypodontia, the more likely the relatives were to also have hypodontia. Additionally, females were more likely to have hypodontia and microdontia, whereas males were more likely to have megadontia and supernumerary teeth and the model was later revised to clarify that both tooth size and shape are involved (Brook et al. 2014). Figure 2.4 shows the aetiological model incorporating all of the multifactorial influences proposed.

Nowadays, most tooth agenesis theories recognise the complex nature of the genetic and environmental interactions involved in hypodontia. In fact, advances in genetic research have made possible the identification and sequencing of the genes involved in tooth morphogenesis, as well as the molecular mechanisms leading to the agenesis of teeth (Parkin et al. 2009). The following discussion will therefore focus on the specific genetic and environmental factors that have so far been implicated in hypodontia.
2.4.3 GENETIC FACTORS

Most craniofacial traits result from a complex interplay between genetic and environmental factors. Heritability can be expressed as a ratio that estimates the extent to which genetic characteristics affect the variation of a trait in a specific population at a specific time, and it is often investigated in twin studies (Harris and Johnson 1991). It can range from 1 (complete genetic control) to zero (complete environmental control; (Harris and Johnson 1991)) but can exceed theoretical thresholds if dominant gene effects and acquired environmental effects are included (Harris and Johnson 1991). Many studies have demonstrated a strong genetic influence in hypodontia. Segregation analyses in many twin and family studies have determined that incisor and premolar hypodontia is inherited via an autosomal dominant gene, with incomplete penetrance and variable expressivity (Ahmad et al. 1998; Alvesalo et al. 1969; Arte 2001; Burzynski and Escobar 1983; Cobourne 2007; Goldenberg et al. 2000; Pirinen et al. 2001; Vastardis 2000; Vastardis et al. 1996). There is no consensus, however, on whether hypodontia is a result of a polygenetic or single gene defect (Larmour et al. 2005), although the former appears to be largely supported in the literature (Brook 1984; Peck et al. 1993; Vastardis 2000).

Since tooth development is under some degree of genetic control, it follows that hypodontia is also under genetic influence. For this reason, recent efforts have focused on identifying the specific genes that are involved in regulating tooth
development. Past research has mainly relied on family studies to identify these genetic variants. Studies of mutant mice and cultured tissue explants have examined the expression of numerous genes involved in tooth development, and provided insight into inductive signalling and hierarchies of downstream transcription factors necessary for tooth development (Jernvall and Thesleff 2000). Over 300 genes are expressed and involved in tooth morphogenesis, including MSX1, PAX9, AXIN2, EDA, SPRY2, TGFA, SPRY4, Wnt10A, FGF3, FGF10, FGFR2, and BMP4 (Alves-Ferreira et al. 2014; Kapadia et al. 2007; Küchler et al. 2013). Among these genes, PAX9 (paired box gene 9), MSX1 (muscle segment homeobox 1), AXIN2 (axis inhibition protein 2) and EDA (ectodysplasin A) are the most frequently reported genes associated with non-syndromic hypodontia (Chishti et al. 2006; Das et al. 2002; De Muynck et al. 2004; Hansen et al. 2007; Li et al. 2008; Mitsui et al. 2014; Mues et al. 2010; Nikopensius et al. 2013). These genes are involved in all major signalling pathways, as well as with the transcription factors mediating the signal transduction cascades (Alves-Ferreira et al. 2014).

PAX9 is a transcription factor expressed in the tooth mesenchyme during tooth morphogenesis (Mitsui et al. 2014), with mutations in this gene being implicated in arresting tooth development at the bud stage. In humans, heterozygous mutations in PAX9 have been associated with non-syndromic hypodontia (Cobourne and Sharpe 2013). Most recently, a case-control study of 306 unrelated Portuguese individuals found that single nucleotide polymorphisms in the PAX9 gene were associated with a high risk of maxillary lateral incisor agenesis (Alves-Ferreira et al. 2014).

MSX1 is a member of a distinct sub-family of homeobox genes; it is expressed in regions of condensing ectomesenchyme in the tooth germ (MacKenzie et al. 1992; Tucker et al. 1998). Mutations in the MSX1 gene have been associated with premature termination of tooth development in animals (Cobourne and Sharpe 2013; Satokata and Maas 1994), and severe forms of hypodontia in humans. Recently, however, a frameshift mutation in MSX1 has been identified in a family demonstrating non-syndromic hypodontia with absence of all second premolars and mandibular central incisors (Kim et al. 2006).

The AXIN2 gene is involved in cell growth, proliferation and differentiation. It is a negative regulator of the Wnt signalling pathway, and this has been associated with
lower incisor agenesis (Callahan et al. 2009; Küchler et al. 2013). In fact, these genes are involved in several forms of tooth agenesis, including syndromes in which tooth agenesis is a regular feature (Nieminen 2009).

More recently, EDA was found to be involved in isolated tooth agenesis. Mutations in this gene cause X-linked hypohidrotic ectodermal dysplasia (HED), which is characterised by sparse hair, fewer and smaller teeth, and a lack of sweat glands (Galluccio et al. 2012). The EDA gene encodes a protein that is part of the tumour necrosis factor (TNF) family of ligands. Several studies have reported sporadic hypodontia in families affected by mutations in EDA and EDA receptor genes (Bergendal et al. 2011; Fan et al. 2008; Tao et al. 2006; Tarpey et al. 2007). EDA has also been shown to be involved in missing maxillary lateral incisor cases (Alves-Ferreira 2014).

Non-syndromic selective tooth agenesis has been shown to either appear sporadically within a member of a family or be inherited (Cobourne and DiBiase 2016). As mentioned, inherited forms follow autosomal dominant, autosomal recessive or autosomal sex-linked patterns of inheritance, with considerable variation in both penetrance and expressivity (Cobourne and DiBiase 2016). As these familial forms represent the most common types of tooth agenesis, a classification according to their genetic basis now exists (Table 2.1).

<table>
<thead>
<tr>
<th>Non-syndromic selective tooth agenesis (STHAG) type</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>STHAG 1 (OMIM 106600)</td>
<td>MSX1 a</td>
</tr>
<tr>
<td>STHAG2 (OMIM 602639)</td>
<td>EDARRAD a</td>
</tr>
<tr>
<td>STHAG3 (OMIM 604625)</td>
<td>PAX9</td>
</tr>
<tr>
<td>STHAG4 (OMIM 150400)</td>
<td>Wnt10A a</td>
</tr>
<tr>
<td>STHAG5 (OMIM 610926)</td>
<td>10q11.2-q21</td>
</tr>
<tr>
<td>STHAG6 (OMIM 613097)</td>
<td>LTBP3</td>
</tr>
<tr>
<td>STHAGX1 (OMIM 313500)</td>
<td>EDA</td>
</tr>
</tbody>
</table>

Table 2.1. Table showing the classification of non-syndromic selective tooth agenesis. Adapted from Cobourne and DiBiase (2016). *These genes are also responsible for syndromic forms of tooth agenesis, including MSX1 (Wiktop syndrome; orofacial clefting and hypodontia); Wnt10A (odonto-onycho-dermal dysplasia); EDA/EDARRAD (hypohidrotic X-linked recessive ectodermal dysplasia).
Craniofacial bones, cartilage, nerves and connective tissue all originate from neural crest cells. Specific developmental cascades are therefore common to the morphogenesis of both teeth and some craniofacial structures (Matalova et al. 2008). Indeed, several syndromes involving hypodontia often exhibit various dysplasias and clefts. Environmental factors have long been known to be associated with a higher risk of some of these craniofacial anomalies. Factors such as trauma, infection and toxins have been implicated (Brook 2009).

Several studies have suggested that intra-uterine conditions could be involved in the aetiology of hypodontia, such as with thalidomide. It was reported that hypodontia was more common in children with thalidomide embryopathy (7.7%) than in normal children (0.4%) (Axrup et al. 1966; Gilbert-Barness 2010). Chemotherapy and radiotherapy treatment in early infancy have also been implicated in the development of hypodontia (Nasman et al. 1997; Nunn et al. 2003). According to some research, rubella infection during pregnancy can cause hypodontia in the developing child (Cameron and Sampson 1996; Parkin et al. 2009). Interestingly, however, maternal health during pregnancy was found to be unrelated to the expression of hypodontia (Boruchov and Green 1971; Parkin et al. 2009). Trauma, such as fracture of the alveolar process, may also contribute to hypodontia, through disruption of tooth germ development (De Coster et al. 2009). However, the evidence in the literature is not sufficient to support this notion.

Neural crest cells are extremely sensitive to high levels of oxidative stress that can arise due to both genetic and environmental factors. It is generally accepted that oxidative stress in the form of smoking, for example (van der Vaart et al. 2004), plays a central role in the development of neural crest cells and the aetiology of craniofacial anomalies. In fact, maternal smoking has been associated repeatedly with a higher risk of CLP (Little et al. 2004). Exposure to alcohol has also been suggested as a risk factor, and, although the evidence has been more inconsistent, some studies have reported that ‘binge’ drinking patterns during pregnancy increase the risk for CLP (Dixon et al. 2011). Given that hypodontia shares similar molecular pathways with some craniofacial anomalies, it would be useful to investigate whether there is an association between environmental factors and
hypodontia. Unfortunately, no study to date has investigated smoking and alcohol as risk factors for hypodontia. Indeed, the identification of environmental risks (particularly if they can be combined with genetic covariates) provides the best opportunity for prevention.

2.5 PSYCHOSOCIAL AND FUNCTIONAL IMPACT

Oral-health-related quality of life (OHRQoL) measures are often used to evaluate the impact of malocclusion on health and well-being. They aim to assess the functional, psychological, and social implications of the condition on an affected individual. Although numerous studies in the literature report on the prevalence, aetiology and treatment of hypodontia, only a handful have investigated OHRQoL in individuals with hypodontia (Meaney et al. 2012). The few studies that have been carried out provide some evidence that hypodontia may have an adverse impact on quality of life.

In a retrospective study of 451 patients with hypodontia, the most common patient complaints included spacing between the teeth, poor aesthetics and awareness of missing teeth (Hobkirk et al. 1994). The authors suggested that delayed referral of the patient is likely to have a negative impact on the social and educational development of these patients. Locker and co-workers reported similar findings, although the affected children had oligodontia (Locker et al. 2010). Interestingly, Laing and colleagues found that the extent of the patients’ complaints was associated with the severity of the condition and the number of missing permanent teeth. Those who had no complaints at the time of presentation had retained primary teeth that masked the problem (Laing et al. 2010).

Functionally, individuals with hypodontia tend to have deeper bites and spaces. Missing posterior teeth may not only result in further deepening of the bite, but the condition may also lead to non-working interferences, poor gingival contours and over-eruption of the opposing teeth (Richardson 1979). Moreover, patients with hypodontia have been found to experience more difficulty in chewing due to a smaller occlusal table. In a recent cross-sectional study, it was found that patients with hypodontia have more difficulty in chewing if the deciduous teeth associated with the missing permanent teeth had been exfoliated (Laing et al. 2010). It is therefore plausible that hypodontia may pose functional limitations that affect an
individual’s general well-being and quality of life in the process, although there is currently limited evidence to support this.

Ultimately, hypodontia carries an aesthetic, functional, psychosocial, and financial burden for affected individuals (Nunn et al. 2003). For these patients, hypodontia is a lifetime problem which requires careful treatment planning in order to ensure best treatment outcomes. Treatment plans also involve long-term maintenance (Hobkirk et al. 2011) and family counselling. The care pathway for patients with hypodontia frequently extends over many years, from initial presentation through to completion of treatment as an adult (Gill et al. 2008).

Most important is the assessment of the complaints of the patients and the parents. Treatment plans needed to manage the missing teeth of hypodontia patients are complex and require an inter-disciplinary approach, which usually comes at a financial cost to both the patient and their family (Hobkirk et al. 2011). For this reason, care is best provided through an experienced team of dental specialists (Goodman 1994; Hobkirk et al. 2011; Nunn et al. 2003).

2.6 SUMMARY

Hypodontia is the most common craniofacial malformation in humans, and it may occur as part of a recognised genetic syndrome or as a non-syndromic isolated trait. The most commonly missing teeth are the mandibular second premolars and the maxillary lateral incisors. While it is not known whether individuals with hypodontia have characteristic skeletal features and growth patterns, several clinical features are commonly seen, including microdontia, transposition of permanent teeth, ectopic permanent teeth and infraocclusion of primary molar teeth. Recent research suggests that both environmental and genetic factors are involved in the aetiology of this condition, with the latter playing a more important role. Finally, it is also likely that specific hypodontia pathways have some effect on the function and psychosocial wellbeing of an individual, given the aesthetic, functional and financial burden for affected individuals.
2.7 STUDY OBJECTIVES

The aims of the present study are to: (1) investigate the association between non-syndromic hypodontia and genetic polymorphisms of candidate genes PAX9, MSX1, AXIN2 and EDA; and (2) examine the association between non-syndromic hypodontia and environmental factors, such as exposure to maternal smoking and alcohol during pregnancy.

2.8 STUDY HYPOTHESES

It was hypothesised that non-syndromic hypodontia is associated with certain genotypes/haplotypes of the genes PAX9, MSX1, AXIN2 and EDA. Furthermore, it was hypothesised that maternal smoking and alcohol intake are associated with hypodontia.
3. CORE METHODS

3.1 RESEARCH APPROACH

A case-control study design was used to investigate specific gene polymorphisms in non-syndromic hypodontia (case) and normal (control) individuals. This study design is the standard method for identifying genetic differences (polymorphisms) underlying complex human traits. The study approach was also well suited for examining whether an association exists between hypodontia and maternal smoking and alcohol intake.

3.2 OVERVIEW OF STUDY DESIGN

Eligible cases were identified from their pre-treatment orthopantomogram (OPG) radiograph and invited to participate in the study. Following the enrolment of cases, controls were recruited from the same pool of orthodontic patients as the cases. Data were collected by means of self-report questionnaires; while DNA samples (in the form of a blood or saliva sample) were collected by a Registered Nurse.

3.3 SAMPLE SELECTION

3.3.1 STUDY PARTICIPANTS AND PHENOTYPE SELECTION

Participants were recruited from previous and existing pools of patients treated in the Orthodontic Clinic at the University of Otago (Dunedin, New Zealand). Eligible patients were offered a free movie voucher as an incentive for participating in the study.

Cases with two specific phenotypes were identified: (1) individuals with one or more missing permanent lateral incisors; and (2) individuals with one or more missing permanent premolars.

3.3.2 INCLUSION CRITERIA

The following inclusion criteria were used:

1. Participants were 9 years of age or older.
2. Presence of a pre-existing good-quality baseline OPG to determine whether there was agenesis of tooth/teeth.
3. Willingness to participate and provide informed consent to take part in the study.

### 3.3.3 EXCLUSION CRITERIA

The same exclusion criteria were applied to both cases and controls, and included:

1. Missing teeth due to previous trauma or extraction.
2. Missing teeth due to a syndromic condition e.g. cleft lip and/or palate, and other craniofacial syndromes.

On-going or previous orthodontic treatment did not preclude participation in this study.

### 3.3.4 SAMPLE SIZE AND STUDY POWER

It was estimated that, with a minor allele frequency of the candidate genes (MSX1, PAX9, AXIN2, and EDA) estimated at 0.3, Type I error set at 5%, and allocating 100 participants to each case/control group, the study will have over 90% power to detect an OR of 2, and 50% power for an OR of 1.5.

### 3.3.5 RECRUITMENT OF CASES

Case recruitment involved clinical and radiographic assessment of patients seeking or receiving orthodontic treatment at the University of Otago Orthodontic Clinic. Existing pre-treatment OPGs were examined by one investigator (AA) to identify missing permanent tooth/teeth, excluding the third molars. A tooth was recorded as congenitally missing if no evidence of the tooth (or its developmental crypt) was found on the radiograph. Treatment records were also checked to confirm that the missing tooth/teeth had not been extracted.

Selected cases were contacted initially by post and invited to participate in the study. This was followed by a phone call during which the purposes of the study were further discussed, and an appointment was arranged if the individual was willing to participate in the study. Information sheets outlining the purpose, nature and design of the study were provided to each participant during this appointment.
and both enrolment and data collection commenced if the participant/parents provided informed consent.

### 3.3.6 RECRUITMENT OF CONTROLS

The recruitment of controls commenced once 75% of the cases were recruited, to ensure that the two groups were comparable for age and sex (frequency-matching). Pre-existing OPGs were again used to assess whether any permanent teeth were missing, excluding the third molars. Controls were recruited from the same pool of orthodontic patients as the cases, and were enrolled in the study in the same manner as the cases.

### 3.4 DATA COLLECTION

The study involved collecting a wide range of data from each participant. Apart from DNA samples, participants were given questionnaires that sought information on socio-demographic characteristics and environmental risk factors.

#### 3.4.1 DNA SAMPLE COLLECTION

Study participants were asked to provide a blood sample in the first instance. They were informed that a saliva sample might be used as an alternative; however, the advantages of more and better quality DNA from a blood sample were emphasised. A Registered Nurse collected blood samples on-site using standard venepuncture procedures. The samples included a 10-ml EDTA tube that was used for DNA preparation and a 5-ml gold top SST tube for serum. The SST vacutainers were centrifuged at 3,500rpm on-site, and then taken to the Merriman Laboratory at the University of Otago for storage. Where venepuncture was refused, 10-ml of saliva was collected instead, using specific saliva kits (DNA genotek\textsuperscript{TM} Oragene-500 kits).

#### 3.4.2 DNA EXTRACTION AND GENOTYPE SEQUENCING

Genomic DNA was extracted from peripheral blood samples using a standard guanidine-HCl based technique and from mouth-swab DNA genotek\textsuperscript{TM} Oragene-500 kits according to the manufacturer’s instructions.
Eighteen single nucleotide polymorphisms (SNPs) from the candidate genes MSX1, PAX9, AXIN2, and EDA were genotyped for both cases and controls. Details on SNP selection and genotyping are presented as supplementary material in Appendices 8.2 and 8.3 respectively.

3.4.3 QUESTIONNAIRES

In addition to providing DNA samples, each participant and his/her mother were required to complete a self-report questionnaire (Appendices 8.4 and 8.5 respectively). These questionnaires included items relating to:

1. Sociodemographic details such as age, gender and ethnicity;
2. Ancestry details of the grandparents, to ensure adequate ethnic matching between the groups; and
3. Environmental pre-natal exposures such as cigarette smoking and alcohol use during pregnancy for the mothers.

The majority of the data was collected at the same time as the DNA samples. However, for those mothers who were unable to attend the one-off appointment, the questionnaire was posted out to them along with a pre-paid return envelope. No time limit was placed on completing the questionnaires, and if clarification was needed about any of the items, assistance was offered without influencing the responses.

3.5 DATA STORAGE

3.5.1 STORAGE OF DNA SAMPLES

All DNA samples obtained during the study were securely stored in such a way that only the investigators of the study were able to gain access to them. These DNA samples will be retained for up to 10 years in the Merriman Labs at the Biochemistry Department at the University of Otago. No other external source, commercial or non-commercial, will have access to any of this information without the permission of the study participants/parents.
3.5.2 STORAGE OF QUESTIONNAIRES

The study participants and their mothers filled out the questionnaires manually; once completed, descriptive information and hard-copy questionnaires were securely stored at the Faculty of Dentistry, University of Otago. Only the investigators involved in the study were able to access these questionnaires.

In order to transfer the collected data into an electronic format, a web-based database was used. This database was specifically developed to improve the efficiency of the entry, storage and management of the data collected from the study participants and their mothers. Different levels of security were implemented to permit various degrees of access to the database, and all sections of the website were protected by unique usernames/passwords. This ensured that the study data were safely stored and not amenable to access or alteration by any third party. In addition, standard security measures were employed to protect against the insertion of rogue coding into the entered data.

3.6 STATISTICAL ANALYSIS

Both genetic and environmental data were analysed using conventional descriptive methods.

Following the generation of descriptive statistics, bivariate analysis was carried out using the Chi-square test or ANOVA as appropriate. Logistic regression analysis was used to adjust for confounding. Analyses were carried out using the Statistical Package for the Social Sciences (SPSS v22.0, SPSS Inc, Chicago ILL), and Stata (version 13.1; Stata Corp LP, College Station, Texas).

3.7 ETHICAL APPROVAL

The study was approved by the University of Otago’s Human Ethics Committee in August 2014 (Reference H14/080). Written and informed consent were collected from all study participants. In addition, parent consent was obtained for study participants under the age of 17 years, Appendices 8.6 and 8.7 contain a copy of the ethics approval; the participants’ information sheet, and the participant/parental consent forms respectively.
3.8 MĀORI CONSULTATION

Consultation with the Ngāi Tahu Research Consultation Committee was sought in May 2014, and their approval was granted in July 2014 (Appendix 8.8).

3.9 FUNDING

The study was supported by grants received from the Sir John Walsh Institute (the Fuller Scholarship) in 2014, and the New Zealand Dental Research Foundation in 2015 and 2016.
4. GENETIC FACTORS AND HYPODONTIA

4.1 INTRODUCTION

Hypodontia, or the agenesis of fewer than 6 permanent teeth, is the most common dental anomaly to affect the human dentition (Nikopensius et al. 2013). Excluding third molars, its prevalence varies from 2.2% to 10.1%, depending on the population studied (Nordgarden et al. 2002; Polder et al. 2004; Rolling and Poulsen 2009), with Europe and Australia having a higher prevalence than North America. Studies have also shown that females are 1.4 times more likely to be affected than males in all three continents (Polder et al. 2004). It is reported that more than 5% of the population lack second premolars or maxillary lateral incisors, making them the most commonly missing teeth after the third molars (Polder et al. 2004; Vastardis 2000).

Hypodontia may occur in association with a genetic syndrome such as cleidocranial dysplasia (Ott et al. 2012), or as a non-syndromic familial form that occurs either sporadically or as a familial trait (Cobourne 2007; Hennekam et al. 2001; Nieminen et al. 1995). The commonest form is the non-syndromic type, and this can follow an autosomal-dominant, autosomal recessive or sex-linked mode of inheritance (Ahmad 1998; Alvesalo 1969; Arte et al. 2001; Cobourne 2007; Goldenberg et al. 2000; Nikopensius et al. 2013; Pirinen 2001; Vastardis 1996). There is a significant amount of variability in the number, position and morphology of the teeth involved, even within affected members of the same family (Nikopensius et al. 2013; Ruiz-Heiland et al. 2016).

Odontogenesis is a highly coordinated reciprocal and progressive process, involving critical stages of interactions and development that occur over a long period of time (Brook 2009; Lee et al. 2014; Mitsui 2014). Numerous signalling molecules, as well as transcription and growth factors, have been identified as regulators of these interactions, which involve multiple genetic signalling pathways between the ectodermal and the neural crest-derived mesenchymal cell layers (Brook 2009; Thesleff 2003; Thesleff and Nieminen et al. 1995). Disturbances of any one of these regulators may alter the number, size, morphology and cytodifferentiation of the teeth (Matalova et al. 2008).
Molecular evidence has shown that the genes msh homeobox 1 (MSX1) and paired box 9 (PAX9), are tooth mesenchymal transcription factors that have key regulatory functions in the initiation and morphogenesis stages of odontogenesis (Jernvall and Thesleff 2000; Thesleff and Nieminen et al. 1995). Findings from animal and human studies have shown that these genes are co-expressed in the dental mesenchyme, and interact during the tooth-bud-to-cap transition (Matalova et al. 2008). In mice, an arrest at an early stage of tooth development results when either one of these genes is homozygously deleted (Kapadia et al. 2007; Kollar and Baird 1969; Mina and Kollar 1987); it has also been shown that both molecules may dimerise and synergistically activate Bmp4 transcription (Kapadia et al. 2007; Ogawa T 2006). According to a recent meta-analysis (Ruf et al. 2013), mutations of PAX9 have been reported in hypodontia families, nine of which are missense mutations (point mutations, in which a single nucleotide change results in a codon that codes for a different amino acid) (Brook 2009); while seven mutations have been associated with agenesis of predominantly second premolars and third molars (De Muynck et al. 2004; Kapadia et al. 2007; Vastardis 1996 ). Meanwhile, MSX1 can cause a slightly different phenotype with more premolar involvement (Chishti et al. 2006; De Muynck et al. 2004), and is also known to have a role in cleft lip and/or palate syndrome (Jumlongras et al. 2001).

The axis inhibition protein 2 (AXIN2) gene is a signalling molecule in the “wingless-type MMTV integration family” (WNT) pathway that is instigated in the early stages of tooth formation (Mues et al. 2010). Mutations in AXIN2, especially autosomal dominant ones, have been shown to cause severe patterns of hypodontia (Callahan et al. 2009; Lammi et al. 2004; Mostowska et al. 2006), and are also involved in both intestinal polyposis and a predisposition to colon and liver cancers (Andrade Filho et al. 2011; Bonds et al. 2014; Galluccio et al. 2012; lavazzo et al. 2016; Mues et al. 2010).

Mutations in the ectodysplasin A (EDA) gene cause X-linked hypohidrotic ectodermal dysplasia (HED), and these mutations not only result in sparse hair and a lack of sweat glands but have been implicated in selective tooth agenesis (Alves-Ferreira 2014; Ayub et al. 2010; Bergendal et al. 2011; Fan et al. 2008; Han et al. 2008; Lee et al. 2014; Li et al. 2008; Mues et al. 2010; Mues et al. 2009). Moreover, several studies have reported sporadic hypodontia in families affected
by mutations in this gene and its receptor (Alves-Ferreira 2014; Ayub et al. 2010; Bergendal et al. 2011; Fan et al. 2008; Han et al. 2008; Li et al. 2008; Mues et al. 2009; Rasool et al. 2008; Tao et al. 2006; Tarpey et al. 2007). According to a review carried out by Galluccio et al. (2012), the probands affected by these mutations were mainly male, and showed tooth agenesis of differing severity without the systemic signs of HED. In these studies, the most commonly missing teeth were molars and premolars (Galluccio et al. 2012), while other studies have shown that EDA primarily affected lateral incisors (Alves-Ferreira 2014; Mues et al. 2010).

Importantly, several studies have shown that nucleotide changes in these genes present not only as rare mutations but also as single nucleotide polymorphisms, and this may represent a low-to-moderate risk for hypodontia (Jobbagy-Ovari et al. 2014; Mostowska et al. 2006; Peres et al. 2005; Wang et al. 2011). The aims of the present study were to investigate the association between non-syndromic hypodontia and genetic polymorphisms of candidate genes PAX9, MSX1, AXIN2 and EDA.
4.2 MATERIALS AND METHODS

4.2.1 PARTICIPANTS

A sample of 360 unrelated individuals (mainly New Zealand Europeans) was recruited for this case-control study. The sample (Table 4.1) included sixty-one cases with one or more missing permanent lateral incisors and/or one or more missing permanent premolars, and 299 controls (individuals with no missing teeth, excluding the third molars). For all cases, tooth agenesis was confirmed both radiographically and clinically in an orthodontic clinic (at the University of Otago, Dunedin, New Zealand). All study procedures were approved by the University of Otago Human Ethics Committee, and all participants provided written informed consent.

Further details of participant recruitment and matching procedures are provided in Section 3. All controls, along with 52 of the cases were also included in the environmental part of the study – as described in Section 5.

4.2.2 CANDIDATE GENE AND SNP SELECTION

Genomic DNA for molecular analysis was extracted from either blood or saliva samples (Section 3 for more details). Selected SNPs from the PAX9, MSX1, AXIN2 and EDA genes were obtained from a review of the literature. Three SNP from the PAX9 gene (rs12881439, rs2073241, rs2073242), 7 SNPs from the MSX1 gene (rs8670, rs12532, rs1042484, rs36059701, rs3775261, rs3821949, rs186861426), 2 SNPs from the AXIN2 gene (rs4128941, rs4791171) and 7 SNPs from the EDA gene (rs1160315, rs12853659, rs2274469, rs2296765, rs2428151, rs2520378, rs62604271) with minor allele frequency (MAF) greater than 10% in the European population were selected using the Human genome build 37 gene chip. Quality control (QC) and imputation of the SNPs was performed as described in Guo et al. (Guo et al. 2014) and Illumina (2016) (Illumina 2016).

More details on SNP selection and genotyping are presented as supplementary material in Appendices 8.2 and 8.3.
4.2.3 STATISTICAL ANALYSIS

Data were analysed using conventional descriptive methods. To compare allele frequencies in cases and controls, cross-tabulations and chi-square tests were used, and odds ratios (ORs) were estimated with 95% confidence intervals (CI).

A logistic regression model was used (with the major allele as the reference category) to evaluate the genotypic associations. Since the EDA gene is located on the X chromosome, the analyses were undertaken with sex taken into account.

Analyses were carried out using the Statistical Package for the Social Sciences (SPSS v22.0, SPSS Inc, Chicago ILL), and Stata (version 13.1; Stata Corp LP, College Station, Texas). For any group of variants that exhibited inter-marker linkage disequilibrium, haplotypes were tested for association using SNP Annotation and Proxy Search program (SNAP v2.2, Broad Institute), which is a program that investigates pairwise linkage disequilibrium.
4.3 RESULTS

The sociodemographic characteristics of the study sample are summarised in Table 4.1. The majority of the participants were female: 50.8% and 61.2% of the cases and controls respectively; and of New Zealand European origin (91.7% and 90.7% of the cases and controls respectively). The total number of missing teeth in the hypodontia group (61 cases) was 141. The frequency of missing second premolars was highest ($n = 80, 56.7$%), followed by the lateral incisors ($n = 56, 39.7$%), of which 52 were maxillary lateral incisors. One individual presented with oligodontia, accounting for the remainder of the 3.6% because, along with agenesis of second premolars, he/she was missing 2 lower central incisors and the upper (and one lower) canines ($n = 5$).

Using an OR of 2 and alpha set at 0.05, the power for the study was 81.0% to 92.2% for detecting an association with PAX9, 35.4% to 90.7% for MSX1, 33.3% to 90.7% for AXIN2 and 63.6% to 93.4% for EDA. Genotypic frequencies in the case and control groups for all selected SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$) (Appendix 8.3.3).

**Table 4.1.** Demographic characteristics of the study sample

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>P value\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n=61)</td>
<td>Controls (n=299)</td>
</tr>
<tr>
<td>Age in years (SD)</td>
<td>17.1 (4.8)</td>
<td>19.3 (7.3)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>31 (50.8)</td>
<td>183 (61.2)</td>
</tr>
<tr>
<td>Male</td>
<td>30 (49.2)</td>
<td>116 (38.8)</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>55 (91.7)</td>
<td>255 (90.7)</td>
</tr>
<tr>
<td>NZ Maori</td>
<td>1 (1.7)</td>
<td>6 (2.1)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (6.7)</td>
<td>20 (7.1)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Chi-square test.

For PAX9, the A-allele of rs2073242 was associated with a high odds (1.49, 95% CI = 1.01-2.21) of having hypodontia ($P = 0.045$); however, this attenuated after adjusting for sex and ethnicity (Table 4.2). No significant differences were found with the remainder of the allelic frequencies of SNPs rs12881439 and rs2073241. For MSX1, the distribution of cases and controls by genotype for the SNPs of
MSX1 gene are presented in Table 4.3. There were no significant differences in allelic frequencies between the two groups. No significant differences were found in allelic and genotypic frequencies between cases and controls for SNPs of the AXIN2 gene, (Table 4.4).

The distribution of cases and controls by genotype for several SNPs of the EDA gene are presented in Table 4.5. There were significant differences in the allelic frequencies between cases and controls for four SNP markers. The T-allele of both rs1160315 and rs12853659 was significantly associated with hypodontia (P < 0.001 and P = 0.002, respectively). The association remained significant after adjusting for ethnicity (P = 0.029), with an OR of 2.79 (95% CI = 1.11-7.01). Meanwhile, it remained only marginally significant after adjusting for sex (P = 0.053), with an OR of 2.44 (95% CI = 0.99-6.03). LD analysis showed that these two SNPs were in high LD (Figure 4.1).

Figure 4.1. Pairwise linkage disequilibrium (LD) analysis of EDA SNPs. LD plot shows that rs1160315 and rs12853659 are in strong LD.
Furthermore, the G-allele of rs2428151 was also significantly associated with tooth agenesis ($P = 0.008$), even after adjusting the OR for sex and ethnicity ($p = 0.043$), with an adjusted OR of $2.87$ (95% CI = 1.04-7.94). Interestingly, the G-allele of rs2520378 showed a protective effect with an OR of $0.61$ (95% CI = 0.38-0.99) and this was significantly different between the cases and controls ($P = 0.049$). However, the effect did not remain significant after the OR was adjusted for sex and ethnicity. A previous study investigated the same EDA SNPs (Alves-Ferreira 2014), and, as such, a meta-analysis of the ORs from both of these studies was carried out. The results of the meta-analysis can be seen in Figure 4.2.
Figure 4.2 Meta-analysis of data collected in the present study and from (Alves-Ferreira 2014). Forest plots for EDA SNPs rs1160315 (A), rs12853659 (B), rs2428151 (C) and rs2520378 (D). I-squared statistics were used to estimate the percentage of variance that is attributable to study heterogeneity.
Table 4.2. Outcomes of the PAX9 gene multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>rs12881439</th>
<th>PAX9</th>
<th>Case genotype, n (%)</th>
<th>Control genotypes, n (%)</th>
<th>Unadjusted</th>
<th>Adjusted(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>G Freq</td>
</tr>
<tr>
<td>rs12881439</td>
<td></td>
<td>43 (70.5)</td>
<td>15 (24.6)</td>
<td>3 (49.2)</td>
<td>21 (17.2)</td>
</tr>
<tr>
<td>rs2073241</td>
<td></td>
<td>26 (42.6)</td>
<td>26 (42.6)</td>
<td>9 (14.8)</td>
<td>44 (36.1)</td>
</tr>
<tr>
<td>rs2073242</td>
<td></td>
<td>20 (32.8)</td>
<td>27 (44.3)</td>
<td>14 (23.0)</td>
<td>55 (45.1)</td>
</tr>
</tbody>
</table>

\(^a\)Adjusted for sex and ethnicity.
Table 4.3. Outcomes of the MSX1 gene multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>MSX1</th>
<th>Case genotype, n (%)</th>
<th>Control genotypes, n (%)</th>
<th>Unadjusted</th>
<th>Adjusted^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>T Freq</td>
</tr>
<tr>
<td>rs8670</td>
<td>45 (73.8)</td>
<td>16 (26.2)</td>
<td>0</td>
<td>16 (13.1)</td>
</tr>
<tr>
<td>rs12532</td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>27 (44.3)</td>
<td>24 (39.3)</td>
<td>10 (16.4)</td>
<td>44 (36.1)</td>
</tr>
<tr>
<td>rs1042484</td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>38 (62.3)</td>
<td>22 (36.1)</td>
<td>1 (1.6)</td>
<td>24 (19.7)</td>
</tr>
<tr>
<td>rs36059701</td>
<td>CC</td>
<td>CG</td>
<td>GG</td>
<td>G Freq</td>
</tr>
<tr>
<td></td>
<td>44 (72.1)</td>
<td>17 (27.9)</td>
<td>0</td>
<td>17 (13.9)</td>
</tr>
<tr>
<td>rs3775261</td>
<td>CC</td>
<td>CA</td>
<td>AA</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>23 (45.1)</td>
<td>21 (41.2)</td>
<td>7 (13.7)</td>
<td>35 (34.3)</td>
</tr>
<tr>
<td>rs3821949</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>40 (65.6)</td>
<td>17 (27.9)</td>
<td>4 (6.6)</td>
<td>25 (20.5)</td>
</tr>
<tr>
<td>rs186861426</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>58 (95.1)</td>
<td>3 (4.9)</td>
<td>0</td>
<td>3 (2.5)</td>
</tr>
</tbody>
</table>

^aAdjusted for sex and ethnicity.
Table 4.4. Outcomes of the AXIN2 gene multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>AXIN2</th>
<th>Case genotype, n (%)</th>
<th>Control genotypes, n (%)</th>
<th>Unadjusted</th>
<th>Adjusted&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4128941</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>57 (93.4)</td>
<td>4 (6.6)</td>
<td>0</td>
<td>4 (3.3)</td>
</tr>
<tr>
<td>rs4791171</td>
<td>TT</td>
<td>TC</td>
<td>CC</td>
<td>C Freq</td>
</tr>
<tr>
<td></td>
<td>11 (18.0)</td>
<td>22 (36.1)</td>
<td>28 (45.9)</td>
<td>78 (63.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adjusted for sex and ethnicity.

<sup>b</sup>Not computable due to large number of dropped cases from analysis.
Table 4.5. Outcomes of the EDA gene multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>EDA</th>
<th>Case genotype, n (%)</th>
<th>Control genotypes, n (%)</th>
<th>Unadjusted Allelic OR (T-Allele, 95% CI)</th>
<th>Adjusted Allelic OR (T-Allele, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1160315</td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>T Freq</td>
</tr>
<tr>
<td></td>
<td>23 (37.7)</td>
<td>11 (18.0)</td>
<td>27 (44.3)</td>
<td>65 (53.3)</td>
</tr>
<tr>
<td>rs12853659</td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>T Freq</td>
</tr>
<tr>
<td></td>
<td>23 (37.7)</td>
<td>12 (19.7)</td>
<td>26 (42.6)</td>
<td>64 (52.5)</td>
</tr>
<tr>
<td>rs2274469</td>
<td>TT</td>
<td>TA</td>
<td>AA</td>
<td>A Freq</td>
</tr>
<tr>
<td></td>
<td>49 (80.3)</td>
<td>8 (13.1)</td>
<td>4 (6.6)</td>
<td>16 (13.1)</td>
</tr>
<tr>
<td>rs2296765</td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>T Freq</td>
</tr>
<tr>
<td></td>
<td>29 (47.5)</td>
<td>17 (27.9)</td>
<td>73 (20.4)</td>
<td>47 (38.5)</td>
</tr>
<tr>
<td>rs2428151</td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>G Freq</td>
</tr>
<tr>
<td></td>
<td>20 (32.8)</td>
<td>12 (19.7)</td>
<td>29 (47.5)</td>
<td>70 (57.4)</td>
</tr>
<tr>
<td>rs2520378</td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>G Freq</td>
</tr>
<tr>
<td></td>
<td>45 (73.8)</td>
<td>9 (14.8)</td>
<td>7 (11.5)</td>
<td>23 (18.9)</td>
</tr>
<tr>
<td>rs62604271</td>
<td>GG</td>
<td>GT</td>
<td>TT</td>
<td>T Freq</td>
</tr>
<tr>
<td></td>
<td>51 (83.6)</td>
<td>5 (8.2)</td>
<td>15 (12.3)</td>
<td>238 (80.1)</td>
</tr>
</tbody>
</table>

*Adjusted for sex and ethnicity.
Hypodontia is a complex dental anomaly that impacts significantly on affected individuals. In order to better understand the underlying mechanism of tooth agenesis, this case-control study was carried out to investigate the relationship between genetic polymorphisms of \textit{PAX9, MSX1, AXIN2} and \textit{EDA} and non-syndromic hypodontia. This particular group of genes was selected because they have already been shown to play a role in the process of hypodontia (Han et al. 2008; Jobbagy-Ovari et al. 2014; Ogawa 2006; Peres et al. 2005; Thesleff and Nieminen et al. 1995; Vieira et al. 2004). The study findings suggest that rs2073242 of the \textit{PAX9}, and rs1160315, rs12853659, and rs2428151 of the \textit{EDA} gene are associated with hypodontia in Caucasians. On the other hand, what may be a protective effect was observed for rs2520378 on the \textit{EDA} gene. No significant associations between non-syndromic tooth agenesis and any of the SNPs of \textit{MSX1} and \textit{AXIN2} genes were observed.

\textit{PAX9} is known to be a transcription factor that is involved in the development of both teeth and the craniofacial complex (Alves-Ferreira 2014; Thesleff and Nieminen et al. 1995). Both mouse and human studies have shown that many different mutations of the \textit{PAX9} gene cause tooth agenesis, and that these mutations must be heterozygous and show an autosomal dominant transmission (Das et al. 2002; Frazier-Bowers 2002; Galluccio et al. 2012; Lammi et al. 2004; Mostowska et al. 2013; Stockton 2008). Stockton et al. reported the first mutation in \textit{PAX9}, where a family tree of a 43-member family revealed that 21 members of the family presented with missing molars mainly, along with missing second premolars and central lower incisors (Stockton 2008). Three other mutations on \textit{PAX9} were reported to be associated with molar agenesis (Das et al. 2002); since then, several studies have been published that identify other mutations in different populations, especially in relation to the agenesis of maxillary lateral incisors (Alves-Ferreira 2014; Jobbagy-Ovari et al. 2014; Kapadia et al. 2007; Suda et al. 2011; Tallon-Walton et al. 2007; Wang et al. 2009a; Wang et al. 2009b; Zhao et al. 2007). Using a relatively large sample of unrelated individuals, a significantly higher proportion of the A-allele of the rs2073242 SNP was found in cases than in controls. Although this association did not remain significant after adjusting for sex and ethnicity, it provides some support for the role of \textit{PAX9} in non-syndromic
tooth agenesis. While other studies have not investigated this particular SNP, significant differences in the genotype frequencies of other SNPs (rs2073246 and rs2073244) have been reported between controls and patients with tooth agenesis (Jobbagy-Ovari et al. 2014; Peres et al. 2005). These SNPs are in high LD with rs2073242 (Jobbagy-Ovari et al. 2014), suggesting that PAX9 polymorphisms play a role in non-syndromic hypodontia.

More recently, molecular animal and human studies have suggested that other genes (such as EDA) are involved in isolated tooth agenesis (Galluccio et al. 2012). The EDA gene, known to cause X-linked HED, has also been implicated in selective tooth agenesis (Alves-Ferreira 2014; Ayub et al. 2010; Bergendal et al. 2011; Fan et al. 2008; Han et al. 2008; Lee et al. 2014; Li et al. 2008; Mues et al. 2010; Mues et al. 2009). The hypodontia phenotype in EDA-associated isolated hypodontia seems to affect the lateral incisors, but other teeth can be involved as well (Mues et al. 2010). The study findings revealed that there were significant differences between cases and controls in allele frequencies for four SNPs of the EDA gene. Cases and controls differed in their frequency of the T-allele of both rs1160315 and rs12853659 (after adjusting for sex and ethnicity). This was also the case for the G-allele of rs2428151, which was significantly associated with tooth agenesis even after adjusting the OR for sex and ethnicity. Interestingly, the G-allele of rs2520378 showed what might be interpreted as a protective effect with an OR of less than 1, although this apparent association did not survive adjusting for sex and ethnicity. These findings were similar to those from a previous study (Alves-Ferreira 2014), and a meta-analysis revealed surprisingly consistent findings from both studies (Figure 4.2).

Another noteworthy finding was the lack of an association between hypodontia and SNPs of the MSXI and AXIN2 genes. Normally, MSXI (which is a homeobox gene) is expressed in dental mesenchyme, but, during the development of the bud cap and bell stages, it is not present in the dental epithelium (Huang et al. 2011; Mackenzie et al. 1991). It is a protein that inhibits the transcription of the target genes through its interaction with other transcription factors such as PAX9. Mutations in MSXI have previously been associated with non-syndromic tooth agenesis in humans (Jobbagy-Ovari et al. 2014; Kim et al. 2006; Lidral and Reising 2002; Mostowska et al. 2012; Pawlowska et al. 2009; Vastardis 1996 ; Vieira et al.
Recent findings suggest that polymorphisms rs8670 and rs12532, which are present in two untranslated regions (intron) of MSX1, could be involved in sporadic and familial tooth agenesis (Pawlowska et al. 2009). One of those polymorphisms (rs12532) was further investigated in a case-control study of 192 hypodontia, 17 oligodontia cases and 260 healthy controls in a Hungarian population (Jobbagy-Ovari et al. 2014). In that same study, rs8670 and rs12532 were also investigated; however, this study showed no significant difference in their genotypes between case and controls (P = 0.069 and P = 0.272 respectively). The sample size was not as large as the aforementioned study, and so there may have been insufficient power to detect an association. Studies have also shown that MSX1 may play a more substantial role in familial cases of tooth agenesis (Kim et al. 2006; Mostowska et al. 2012; Vieira et al. 2004) and, given that our sample consisted of unrelated individuals, the findings suggest that MSX1 may play a less important role in sporadic forms of hypodontia.

There is relatively less evidence for the association between AXIN2 and non-syndromic hypodontia, although the findings of a handful of studies suggest that an association may exist (Bergendal et al. 2011; Callahan et al. 2009; Jobbagy-Ovari et al. 2014; Lammi et al. 2004; Mostowska et al. 2006; Wang et al. 2011). The AXIN2 gene is a negative regulator of the WNT signalling pathway and is expressed in the enamel knot, the dental mesenchyme of the dental papilla, and in mesenchymal odontoblasts during mice odontogenesis (Lammi et al. 2004). It was first implicated in the process of tooth agenesis when, in a four-generation Finnish family with severe familial oligodontia, no mutations in MSX1 and PAX9 genes were detected; instead, the presence of a mutation in the AXIN2 gene was noted (Lammi et al. 2004). The association between this gene and tooth agenesis was confirmed in two later studies (Callahan et al. 2009; Mostowska et al. 2006). In 2013, a cross-sectional comparison of 82 individuals with tooth agenesis and 328 individuals with no defects observed an association between lower incisor agenesis and rs2240308 on the AXIN2 gene (Küchler et al. 2013). In this study, however, the genotypes of the two polymorphisms investigated (rs4128941 and rs4791171) did not differ significantly between individuals with tooth agenesis and those in the control group.
The current study had some limitations. First, a full phenotypic investigation, such as analysis of hair follicle patterns or other traits, was not carried out to ensure that all the cases were in fact “non-syndromic”. Cases were deemed non-syndromic based on the assessment of extra-oral clinical features by the author. Therefore, it cannot be definitively excluded that some of the cases may have presented with micro-manifestations of a syndrome such as ectodermal dysplasia. Second, the small number of cases means that the study was not powered to detect associations with rare alleles as well as ones with around an OR of 2.0, and meant that probably alleles with a major effect were detected. Third, the sample size was too small to test for interactions between genetic and environmental factors (such as maternal smoking). Fourth, investigating a large number of SNPs meant that there was a risk of false negatives, although this was negated to some extent by increasing the number of controls (matching one case to three controls). Fifth, a sub-analysis for different patterns of hypodontia could not be carried out because both agenesis of lateral incisors and permanent premolars had to be combined due to the relatively small sample size. Finally, population stratification is an important concern in case-control candidate-gene association studies (Cardon and Palmer 2003; Thomas and Witte 2002), including in this study. Population stratification arises when cases and controls have different allele frequencies that are attributable to diversity in background population rather than to outcome status (Cardon and Palmer 2003). In this study, this could have been mitigated via careful selection of controls so that they match cases in terms of ethnicity (Thomas and Witte 2002).

4.5 CONCLUSIONS

This study confirms that polymorphisms of the PAX9 and EDA genes may play a significant role in the aetiology of tooth agenesis. Moreover, the rs2520378 SNP of the EDA gene may protect against the development of hypodontia. No significant associations between any of the studied SNPs on MSX1/AXIN2 genes and non-syndromic tooth agenesis were found. Although the exact molecular mechanisms involved in tooth agenesis remain unknown, the findings add to the understanding of the genetic mechanisms underlying non-syndromic forms of hypodontia.
5. ENVIRONMENTAL FACTORS AND HYPODONTIA

5.1 INTRODUCTION

Tooth agenesis is the most common developmental defect in the permanent dentition, with at least 200 million humans around the world failing to develop at least one tooth (Karadas et al. 2014; Yin and Bian 2015). This number is higher when the third molars are included. Hypodontia, where fewer than 6 teeth are missing, is the most common form (Nunn et al. 2003; Yin and Bian 2015), with the mandibular and maxillary second premolars and maxillary lateral incisors being the most commonly missing teeth (Bailleul-Forestier et al. 2008; Polder et al. 2004).

Agenesis occurs during the initiation phase of tooth development due to a breakdown of communication between mesenchymal tissue and the overlying epithelium (Thesleff 2003), both originating from the neural crest.

Although the subject of many investigations, the aetiologic factors involved in tooth agenesis remain largely unknown although, it is well established that genetic variation plays a major role (Galluccio et al. 2012; Kindelan et al. 1998). Indeed, a number of studies implicated mutations in genes such as MSX1, PAX9 and AXIN2 and EDA in familial forms of non-syndromic hypodontia (Lammi et al. 2004; Stockton 2008; Vastardis 1996). Nevertheless, there is compelling evidence that conditions resulting from defects in tooth morphogenesis (including hypodontia) are caused by a complex combination of genetic and environmental factors (Krauss and Hong 2016).

Tooth development can be disturbed by a number of different factors (Nieminen 2009). These include trauma to the alveolar process or jaw, jaw surgery or iatrogenic damage to the developing tooth germ from traumatic extraction of the overlying primary tooth, all of which can cause disruption to normal tooth development (Grahen 1956; Nunn et al. 2003). Some infections during pregnancy, such as rubella, have been reported to cause hypodontia in the developing child (Cameron 1996; Parkin et al. 2009). Interestingly, maternal ill-health during pregnancy was found to be unrelated to hypodontia (Boruchov 1971; Parkin et al. 2009). It has also been reported that tooth agenesis is more common in children with thalidomide embryopathy (7.7%) than in children in the wider population.
(0.4%) (Axrup K 1966), while a higher prevalence of tooth agenesis was observed among people exposed to dioxin in Seveso, Italy (Alaluusua et al. 2004). Similarly, the sensitivity of tooth development to childhood anti-cancer treatment (by radiotherapy, chemotherapy or stem cell transplantation) in early infancy has also been implicated in the development of hypodontia (Dahllöf and Huggare 2004; Holtta et al. 2005; Nasman 1997; Nunn et al. 2003). Whatever the cause is, it seems that a disruption to the molecular pathways during the early stages of tooth development occurs which results in tooth agenesis.

Smoking and alcohol use are recognised risk factors for developmental disorders in general, while exposures such as cancer therapy or other drugs have been implicated in some conditions. Surprisingly, the influence of maternal tobacco smoking and alcohol consumption on tooth development has not been investigated.

Almost one-third of the world’s population aged 15 years or older (including some 12% of women) smoke cigarettes (Evans et al. 1979), while, in developed countries, the prevalence in women is estimated to be up to 24% (Evans et al. 1979). Maternal smoking has long been associated with a higher risk of birth defects such as cleft lip and/or palate (CL/P), with the odds of having children with CL/P among mothers who smoke almost 1.3 times those who do not (Dixon 2011; Little 2004; Shi et al. 2007; Shi et al. 2008). Given that maternal smoking is strongly associated with CL/P, it is prudent to investigate its association with hypodontia.

Alcohol is widely recognised as a human teratogen (Gilbert-Barness 2010; Mead and Sarkar 2014), while maternal alcohol consumption is associated with fetal alcohol spectrum disorders (Krauss and Hong 2016). Moreover, alcohol has been associated with various craniofacial defects and holoprosencephaly (HPE) (Krauss and Hong 2016), because foetal alcohol exposure induces craniofacial anomalies and strain-dependent HPE in mice (Downing et al. 2009; Hong and Krauss 2012). Exposure to maternal alcohol consumption has also been proposed as a risk factor for the development of CL/P (Mossey and Little 2009), although substantiated links to alcohol consumption have yet to be confirmed (Dixon 2011).
No studies have investigated the association between non-syndromic hypodontia and environmental factors such as maternal smoking and alcohol consumption during pregnancy. Because of the known adverse effects of tobacco smoking and alcohol consumption on reproductive health and health in general (as well as the genetic mutations and pathways hypodontia and other birth defects such as CL/P share), it can be expected that an association exists between these factors and hypodontia.

The aims of this chapter were to test the hypothesis of a positive association between non-syndromic hypodontia and environmental factors, such as exposure to smoking and alcohol during pregnancy.
5.2 MATERIALS AND METHODS

5.2.1 PARTICIPANTS

Patients were recruited from previous and existing pools of patients treated in the orthodontic clinic at the University of Otago (Dunedin, New Zealand) from August 2014 until December 2015. The sample comprised of 89 cases with one or more missing permanent lateral incisors and/or one or more missing permanent premolars. These cases were frequency matched to 253 controls (patients with no missing teeth, excluding the third molars). All study procedures were approved by the University of Otago Human Ethics Committee, and all participants provided written informed consent. Sample size estimation was based on the minor allele frequencies of the candidate genes (MSX1, PAX9, AXIN2, and EDA), which are reported in Section 4.

Further details of participant recruitment and matching procedures are provided in Section 3.

5.2.2 SELF-REPORT MEASURES

Study participants and their mothers were asked to attend a one-off appointment to complete questionnaires. A questionnaire sought information relating to sociodemographic details such as age, gender, ethnicity and ancestry.

Information on maternal exposures to risk factors during pregnancy was collected using maternal self-report. Information about maternal sociodemographic characteristics, smoking habits (including exposure to second-hand smoking), the number of cigarettes smoked per day, and alcohol and caffeine consumption during pregnancy was also collected. In addition, information about gestational age was collected, with this grouped into three ordinal categories: “early” if the baby was born before 37 weeks; “full-term” if born at 37-40 weeks; and “late” if born after 40 weeks. Questionnaires were posted out with a pre-paid return envelope to the mothers who were unable to attend the appointment.

5.2.3 STATISTICAL ANALYSIS

Data were analysed using conventional descriptive methods. Univariate and multivariate regression procedures were applied to estimate the associations
between maternal smoking, alcohol and caffeine exposure during pregnancy and non-syndromic hypodontia. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using logistic regression.

The regression models were adjusted for the following potential confounders: maternal age at delivery; sex and gestational age of the child; and household socio-economic background. Household socio-economic status was determined using the New Zealand Socio-economic Index 2006 (Milne et al. 2013). Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS v22.0, SPSS Inc, Chicago ILL).
5.3 RESULTS

5.3.1 PATTERN OF AGENESIS
Most of the cases (n = 47, 52.8%) had agenesis of one or more permanent premolars, while approximately one-third had agenesis of the permanent lateral incisors (n = 29, 32.6%). A combination of premolar and lateral incisor agenesis was observed in 12 cases (13.5%), while one individual (1.1%) was missing these teeth in combination with the lower second molars.

5.3.2 SOCIODEMOGRAPHIC CHARACTERISTICS OF SAMPLE
The sociodemographic characteristics of the study sample are summarised in Table 5.1. Cases had a mean age of 15.9 years (SD = 5.1), while controls had a mean age of 16.9 years (SD = 7.9). The majority of both the cases and controls were female (57.3% and 60.5% respectively). There were no significant differences between the study groups for any of the sociodemographic characteristics.
Table 5.1. Overview of sociodemographic characteristics of hypodontia cases and controls (percentages are column percentages).

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th></th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n=89)</td>
<td>Controls (n=253)</td>
<td></td>
</tr>
<tr>
<td>Participants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age in years (SD)</td>
<td>15.9 (5.1)</td>
<td>16.9 (7.9)</td>
<td></td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>51 (57.3)</td>
<td>153 (60.5)</td>
<td>0.600</td>
</tr>
<tr>
<td>Male</td>
<td>38 (42.7)</td>
<td>100 (39.5)</td>
<td></td>
</tr>
<tr>
<td>Gestation age (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>26 (29.2)</td>
<td>76 (30.0)</td>
<td>0.833</td>
</tr>
<tr>
<td>Full-term</td>
<td>28 (31.5)</td>
<td>83 (32.9)</td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td>25 (28.0)</td>
<td>62 (24.5)</td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethnicity (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>67 (75.3)</td>
<td>191 (75.5)</td>
<td>0.803</td>
</tr>
<tr>
<td>NZ Maori</td>
<td>2 (2.2)</td>
<td>4 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>9 (10.1)</td>
<td>20 (7.9)</td>
<td></td>
</tr>
<tr>
<td>Socio-economic status (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.517</td>
</tr>
<tr>
<td>High (1-2)</td>
<td>27 (30.3)</td>
<td>67 (26.5)</td>
<td></td>
</tr>
<tr>
<td>Medium (3-4)</td>
<td>39 (43.8)</td>
<td>110 (43.5)</td>
<td></td>
</tr>
<tr>
<td>Low (5-6)</td>
<td>5 (5.6)</td>
<td>23 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Age at delivery (%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.491</td>
</tr>
<tr>
<td>&lt;20 years</td>
<td>3 (3.4)</td>
<td>9 (3.6)</td>
<td></td>
</tr>
<tr>
<td>20-30 years</td>
<td>27 (30.3)</td>
<td>74 (29.2)</td>
<td></td>
</tr>
<tr>
<td>30-40 years</td>
<td>42 (47.2)</td>
<td>105 (41.5)</td>
<td></td>
</tr>
<tr>
<td>&gt;40 years</td>
<td>4 (4.5)</td>
<td>23 (9.1)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Chi-square test. Missing data: <sup>b</sup>10 (11.3%) cases, 32 (12.6%) controls; <sup>c</sup>11 (12.4%) cases, 38 (15.0%) controls; <sup>d</sup>18 (20.2%) cases, 53 (20.9%) controls; <sup>e</sup>13 (14.6%) cases, 42 (16.6%) controls.
5.3.3 MATERNAL EXPOSURES TO RISK FACTORS DURING PREGNANCY

Maternal exposures to risk factors during pregnancy are summarised in Table 5.2. The proportion of mothers reporting smoking was higher in the hypodontia than in the (P = 0.009). The frequency of smoking (that is, the number of cigarettes per day) also differed between the groups, and showed a biological gradient with 50% (n=9) of mothers reporting smoking heavily during pregnancy (> 10 cigarettes per day) having children with hypodontia (P = 0.031). Maternal exposure to second-hand smoking did not differ between the two groups (P = 0.555). A higher proportion of mothers of hypodontia individuals than controls reported drinking more than one glass of alcohol per week during pregnancy, although, this difference was not statistically significant (P = 0.109). In addition, no significant difference was found between cases and controls in maternal caffeine consumption during pregnancy.

Table 5.2. Maternal smoking habits and consumption of alcohol and caffeine during pregnancy (percentages are column percentages).

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases (n=89)</th>
<th>Controls (n=253)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking habits (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>61 (68.5)</td>
<td>199 (78.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>Yes</td>
<td>18 (20.2)</td>
<td>22 (8.7)</td>
<td></td>
</tr>
<tr>
<td>Number of cigarettes/day (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>61 (68.5)</td>
<td>199 (78.7)</td>
<td></td>
</tr>
<tr>
<td>1-9</td>
<td>9 (10.1)</td>
<td>13 (5.1)</td>
<td>0.031</td>
</tr>
<tr>
<td>&gt;10</td>
<td>9 (10.1)</td>
<td>9 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Second-hand smoking (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>65 (73.0)</td>
<td>175 (69.2)</td>
<td>0.555</td>
</tr>
<tr>
<td>Yes</td>
<td>14 (15.7)</td>
<td>46 (18.2)</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>64 (71.9)</td>
<td>195 (77.1)</td>
<td>0.109</td>
</tr>
<tr>
<td>&gt; 1 glass/week</td>
<td>15 (16.8)</td>
<td>26 (10.3)</td>
<td></td>
</tr>
<tr>
<td>Caffeine consumption (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29 (32.6)</td>
<td>76 (30.0)</td>
<td>0.711</td>
</tr>
<tr>
<td>Yes</td>
<td>50 (56.2)</td>
<td>145 (57.3)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Chi-square test. <sup>b</sup>Missing data; <sup>b</sup>10 (11.3%) cases, 32 (12.6%) controls.
Table 5.3 summarises the unadjusted and adjusted odds ratios for hypodontia. Maternal smoking during pregnancy was associated with a higher risk of having a child with hypodontia (2.05 ≤ adjusted OR ≤ 4.18). Maternal alcohol consumption during pregnancy was not associated with hypodontia in the child (P = 0.109). This was also true for gestational age and maternal consumption of caffeine during pregnancy (P ≥ 0.711). Post-hoc power analysis for testing the association between maternal alcohol consumption and hypodontia ranged from 37.9% to 88.2%. In addition, the children of older mothers and lower-SES mothers were no more at risk.

Table 5.3. Unadjusted and adjusted odds ratios for hypodontia

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>P value</th>
<th>Adjusted</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio (95%CI)</td>
<td></td>
<td>Odds Ratio (95%CI)</td>
<td></td>
</tr>
<tr>
<td>Number of cigarettes/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1-9</td>
<td>2.26 (0.92-5.54)</td>
<td>0.075</td>
<td>2.05 (0.73-5.75)</td>
<td>0.232</td>
</tr>
<tr>
<td>&gt;10</td>
<td>3.26 (1.24-8.58)</td>
<td>0.017</td>
<td>4.18 (1.49-11.80)</td>
<td>0.007</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>1.76 (0.88-3.52)</td>
<td>0.109</td>
<td>1.84 (0.83-4.08)</td>
<td>0.132</td>
</tr>
<tr>
<td>Caffeine consumption</td>
<td>0.90 (0.53-1.54)</td>
<td>0.711</td>
<td>0.89 (0.48-1.64)</td>
<td>0.700</td>
</tr>
<tr>
<td>Maternal age at delivery</td>
<td>1.01 (0.96-1.05)</td>
<td>0.800</td>
<td>1.01 (0.96-1.07)</td>
<td>0.622</td>
</tr>
<tr>
<td>Female</td>
<td>1.14 (0.70-1.86)</td>
<td>0.600</td>
<td>1.32 (0.73-2.39)</td>
<td>0.358</td>
</tr>
<tr>
<td>Socio-economic status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (1-2)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Medium (3-4)</td>
<td>0.88 (0.49-1.57)</td>
<td>0.664</td>
<td>0.84 (0.45-1.56)</td>
<td>0.575</td>
</tr>
<tr>
<td>Low (5-6)</td>
<td>0.54 (0.19-1.57)</td>
<td>0.256</td>
<td>0.49 (0.16-1.56)</td>
<td>0.228</td>
</tr>
<tr>
<td>Gestation age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On-time</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Early</td>
<td>1.01 (0.55-1.88)</td>
<td>0.965</td>
<td>0.84 (0.40-1.76)</td>
<td>0.646</td>
</tr>
<tr>
<td>Late</td>
<td>1.20 (0.64-2.25)</td>
<td>0.580</td>
<td>1.15 (0.59-2.24)</td>
<td>0.692</td>
</tr>
</tbody>
</table>

*aAll variables entered into the model.*
5.4 DISCUSSION

This study examined the association between non-syndromic hypodontia and common environmental factors such as exposure to maternal smoking and alcohol and caffeine consumption during pregnancy. The findings suggest that (1) maternal cigarette smoking during pregnancy is significantly associated with hypodontia; and, (2) a biological gradient was apparent, with the consumption of 10 or more cigarettes per day during pregnancy associated with greater odds of having a child with hypodontia. The lack of similar studies makes it difficult to compare these findings with previous literature. Instead, this body of work provides new evidence to support the hypothesis that maternal smoking during pregnancy is a risk factor for having a child with hypodontia.

The aetiology of hypodontia has often been attributed to both genetic and environmental factors (Parkin et al. 2009); however, an association between this developmental condition and common environmental risk factors has not been previously reported. Investigations into this aspect of tooth agenesis are of utmost importance, since environmental risk factors may present the best possibility for developing preventive measures.

Craniofacial bones, cartilage, nerves and connective tissues all originate from neural crest cells. Specific developmental cascades are therefore common to the morphogenesis of both teeth and some craniofacial structures (Matalova et al. 2008), and indeed, several syndromes involving hypodontia often exhibit various dysplasias and clefts. Environmental factors have long been known to be associated with a higher risk of some of these craniofacial anomalies. Factors such as trauma, infections and toxins have been implicated (Brook 2009). Neural crest cells are extremely sensitive to high levels of oxidative stress that can arise due to both genetic and environmental factors (Morgan 2008; Sakai 2016). It is generally accepted that oxidative stress, in the form of smoking for example (van der Vaart 2004), plays a central role in the pathogenesis of neural crest cells disorders and the aetiology of craniofacial anomalies. In fact, maternal smoking and alcohol consumption during pregnancy have long been implicated with a higher risk of craniofacial deformities such as CL/P (Chung et al. 2000; DeRoo et al. 2016; Dixon 2011; McKinney et al. 2016; Mossey and Little 2009). Despite the common genetic
pathways shared between CL/P and hypodontia, and the fact that these environmental exposures are relatively common, no study to date has tested a possible association between hypodontia and maternal smoking and alcohol use during pregnancy.

Apart from its novelty, the study has several strengths. First, adjustment for confounding factors associated with cigarette smoking, such as alcohol consumption (Chung et al. 2000) were made. All individuals with any other associated congenital anomalies and/or syndromes (such as CL/P) were also excluded in order to avoid confounding of the association between cigarette smoking and hypodontia. Second, the study findings indicated a biological gradient with high odds of having newborns with hypodontia with increased maternal cigarette smoking during pregnancy. This is important, as a biological gradient effect is known to be a criterion of causation of a disease by an exposure (Chung et al. 2000; Hill 1965; Trout 1981). Third, other risk factors were also investigated, apart from smoking, such as maternal consumption of alcohol and caffeine during pregnancy. Maternal consumption of alcohol during pregnancy is known to be associated with having children affected by foetal alcohol syndrome or craniofacial anomalies (DeRoo et al. 2016; Krauss and Hong 2016). Interestingly, no significant association between these environmental factors and hypodontia was observed. One plausible reason for this could be due to the limited power of the testing used. This could be a consequence of under-reporting as a result of the stigma surrounding maternal alcohol consumption during pregnancy. For caffeine consumption, the findings were consistent with the existing literature where caffeine has repeatedly been shown to have no association with congenital anomalies or the overall health of newborns (Browne 2006; Christian and Brent 2001; Golding 1995; Nawrot et al. 2003).

The present study has several potential limitations. First, the self-reported exposure data could be influenced by recall bias, and this may have affected the validity of the data. Second, healthy individuals with no missing teeth were allocated to the control group so that the findings may be generalised to the general population. However, this introduces differential recall bias, since cases and their mothers may recall their exposure more vividly than the healthy controls, because they are more affected by the deformity of interest and have had longer
to consider to possible causes (Hennekens 1987). As a result, cases may report greater exposure, and this would in turn inflate the odds ratio and bias against the null hypothesis. On the other hand, guilt or social-desirability bias in mothers of hypodontia cases may prompt them to under-report the smoking or alcohol exposures during pregnancy, resulting in an underestimation of the odds ratio and so favouring the null hypothesis. These limitations could be addressed by allocating individuals with other congenital anomalies that have not been found to have an association with smoking exposure to a second group of controls. This would potentially minimise recall bias, since mothers of both cases and controls should recall their smoking exposure similarly. Alternatively, a prospective study design may also address this limitation, given that the outcome is not very rare. In terms of smoking history, the main limitation in the questionnaire was that it did not collect details on the temporality and duration of smoking. Third, the study involved exploration of the associations in a clinic-based sample. These associations may not be generalisable to the whole community because the clinical cases may have different characteristics from community cases. Finally, another limitation is the relatively high number of missing cases, which may have skewed the results.

5.5 CONCLUSIONS

In conclusion, there was an association between hypodontia and maternal smoking during pregnancy. There is a biological gradient effect to this association, and this remained significant even after adjusting for confounders. These findings establish a platform for future research in this area, and will add to understanding of the environmental influences on non-syndromic hypodontia.
6. DISCUSSION

The study was carried out in two parts in order to: (1) investigate the association between non-syndromic hypodontia and single nucleotide polymorphisms (SNPs) of candidate genes paired box 9 (PAX9), msh homeobox 1 (MSX1), axis inhibition protein 2 (AXIN2), and ectodysplasin A (EDA); and (2) examine the association between non-syndromic hypodontia and environmental factors, such as exposure to smoking and alcohol during pregnancy. The first part involved a sample of 360 unrelated individuals, and included 61 cases with one or more missing permanent lateral incisors and/or one or more missing permanent premolars, and 299 controls (individuals with no missing teeth, excluding the third molars). The second part involved 89 cases with the same phenotype as above, and these were frequency-matched to 253 controls. Self-report data from both the participants and their mothers were collected, while DNA samples (in the form of blood or saliva) were collected from participants.

After adjusting for sex and ethnicity, the T-allele of rs12853659 (EDA) was associated with a higher risk of hypodontia (odds ratio, OR = 2.79, P = 0.029), as was the G-allele of rs2428151 (EDA) (OR = 2.87, P = 0.043). The A-allele of rs2073242 (PAX9) was associated with high odds (1.49, 95% CI = 1.01-2.21) of having hypodontia (P = 0.045); however, this attenuated after adjusting for sex and ethnicity. No statistically significant associations were found with the AXIN2 and MSX1 genes. Analysis of the environmental data revealed a positive association between hypodontia and maternal cigarette use during pregnancy, as well as a biological gradient effect with the number of cigarettes smoked per day.

6.2 STRENGTHS OF THE STUDY

There is increasing evidence to suggest that both genetic and environmental factors are implicated in the aetiology of non-syndromic hypodontia (Brook 2009; Cobourne 2007; Galluccio et al. 2012; Matalova et al. 2008; Townsend et al. 2009). However, one problem with previous studies is that most utilise family designs, which are not ideal for investigating complex family traits such as tooth agenesis. Findings from the few case-control studies published to date have not been replicated or verified. Moreover, most of those case-control studies have
investigated oligodontia or molar agenesis, with few using well-defined phenotypes such as second premolar or lateral incisor agenesis. Finally, previous studies have not investigated the role of environmental factors, such as maternal cigarette smoking, in hypodontia.

The present study addressed these shortcomings and, consequently, had a number of strengths. First, a case-control study design was used, allowing investigation of both the genetic and environmental factors that may be involved with hypodontia. Second, this is the first study to identify a possible association between non-syndromic hypodontia and maternal smoking during pregnancy. Third, the study sample size estimation was based on the minor allele frequencies of the selected candidate genes (MSX1, PAX9, AXIN2, and EDA), and the study was adequately powered to detect genes with relatively small-moderate effects. Fourth, the genes investigated have been shown to be associated with non-syndromic hypodontia (Han et al. 2008; Jobbagy-Ovari et al. 2014; Ogawa 2006; Peres et al. 2005; Thesleff and Nieminen et al. 1995; Vieira et al. 2004); as such, the genetic findings from this study will add to understanding of the genetic mechanisms involved in tooth agenesis. Indeed, the genes analysed in this study could be regarded as candidates for mutation detection in individuals with hypodontia, and may prove useful in screening hypodontia in the future. Finally, multivariate analysis was used in order to adjust for possible confounding in both the genetic and environmental parts of the study.

6.3 LIMITATIONS OF THE STUDY

As previously mentioned, the study had several limitations, in both the genetic and environmental aspects.

The genetic aspect of the study had the following limitations. First, a substantial number of the saliva samples had poor DNA yield. This meant that a number of the cases had to be dropped during the genotyping process, resulting in fewer cases than originally recruited. The use of buccal swabs rather than saliva collection in a tube may have been more useful. Second, the small number of cases meant that the study was not powered to detect associations with rare alleles. As well as this, a small case sample introduces a risk of false negatives, although this was negated to some extent by matching every one case to three controls. The
interpretation of the genetic findings is also limited by the sample size, and in order
to further substantiate these observations, analyses involving a larger sample size
and advanced methods (such as genome-wide association studies) are required.
Finally, a full phenotypic investigation, such as analysis of hair follicle patterns or
other traits, was not carried out to ensure that all the cases were in fact “non-
syndromic”.

Similarly, some limitations exist in the environmental aspect of the study. First, a
number of the self-report questionnaires were incomplete or contained fields that
were incorrectly marked, resulting in missing data. The missing data involved both
sociodemographic details (such as ethnicity) and maternal smoking history during
pregnancy. Second, because this is a case-control study, the assessments of
exposure were retrospective and so are susceptible to recall bias, which may have
affected data validity. Third, given the well-publicised negative connotations of
exposures such as tobacco smoking in general and alcohol consumption during
pregnancy, mothers may well have under-reported these exposures when
completing the questionnaires. This would result in underestimation of the odds
ratio and favouring of the null hypothesis. Perhaps including the biological fathers
during collection of the self-report data would have aided in validating the
responses given by the mothers. Fourth, the associations between hypodontia and
the environmental risk factors were explored in a clinic-based sample, and both
the cases and controls were primarily New Zealand Europeans. This means that
the findings may not be generalisable to individuals from the wider community or
those with different ethnic backgrounds.

6.4 FUTURE RESEARCH DIRECTIONS

The findings from this case-control study suggest that both genetic factors and
environmental exposures such as maternal smoking during pregnancy are involved
in the aetiology of non-syndromic hypodontia.

The role of genetic differences is well documented in the hypodontia literature;
however, to further verify the role of genetic polymorphisms in hypodontia, larger
sample sizes and more advanced methods (such as genome-wide association
studies) are required. Indeed, the discovery of associations between genetic
polymorphisms in tooth development and non-syndromic hypodontia in different
populations will further enhance understanding of the genetic and molecular mechanisms involved in both normal and abnormal tooth development.

Conversely, the role of environmental risk factors in hypodontia is less known and has been only rarely investigated. More studies are required to replicate and verify these findings, especially with respect to the role of maternal smoking and alcohol consumption during pregnancy in tooth agenesis. In addition, prospective studies with well-quantified measures of exposure are needed to establish whether any of the observed associations are causal.

Most importantly, more studies are required to investigate the role of epigenetics. Epigenetics attempts to explain changes in gene expression without nucleotide sequencing alterations (Townsend et al. 2005). Waddington originally proposed this field of research; and he linked it to methods by which genotype gives rise to phenotype (Waddington 1942; Waddington 1957). In a broader sense, epigenetics portray interactive processes that occur between cells at during dental development, as well as those processes that operate directly on DNA (Townsend et al. 2015). Epigenetic effects are at times deemed a “third source of developmental differences” that, in addition to both genetic and environmental factors, can account for phenotypic variation in development (Molenaar et al. 1993). Townsend et al. (2015) consider that a multifactorial model – with genetic, epigenetic and environmental influences – provides the best explanations for observations involving hypodontia in mono-zygotic twins, for example, who have same genotypes but display different dental phenotypes (Townsend et al. 2015; Brook et al. 2002; Brook 1984) rendering the monogenic mode of inheritance too simplistic. Therefore, the field of epigenetics should be further studied in order to further enhance the understanding of hypodontia, as well as its relationship with environmental and genetic make-up of affected individuals. Furthermore, research into any potential gene-environment interactions is also needed to investigate which specific environmental factors interact with specific genetic variants that predispose to hypodontia. Moreover, the realisation of regenerative therapies for missing teeth will also be more likely with deeper knowledge of the genes involved in tooth development.
6.5 CLINICAL IMPLICATIONS

Hypodontia is by far the most common form of congenital tooth absence and can involve a variable number of teeth. Identification of the gene polymorphisms responsible for tooth agenesis and quantifying the exact role of environmental and epigenetic factors in regulating tooth development has important clinical implications. The treatment of hypodontia is challenging, and centres on improving both aesthetics and function. It often involves a multidisciplinary approach, and this includes the provision of different types of prostheses that are expensive and need lifetime maintenance. The identification of environmental risk factors – particularly if they can be personalised with genetic information – may provide the best short-term opportunity for both personalised and population-level prevention.

It is hoped that better knowledge of the molecular pathways involved in tooth development – along with the rapid advances in stem cell research – will facilitate the exciting prospect of tooth bioengineering. Currently, the approach is to generate tooth substitutes from autologous human tissues, and this might be an alternative to replacement of missing teeth in hypodontia patients. Tissue engineering is promising (Vacanti et al. 2001) and has been used in maxillofacial surgery to generate mandibular condyles in vitro (Abukawa et al. 2003). Moreover, advances in molecular studies have introduced experimental approaches with recombinant protein therapy. This is especially the case with recombinant-EDA, whereby short-term recombinant protein therapy has been explored as a therapy to permanently correct a developmental genetic defect. Gaide and Schneider (2003) provided the first working example of recombinant therapy. They concluded that recombinant therapy rescued the phenotype (hypohidrotic ectodermal dysplasia – HED) in the offspring of Tabby mice, since the jaw and molars of the mice regained both their normal sizes and their pattern of sharp cusps (Gaide and Schneider 2003). Another more recent study has demonstrated both reversions of oligodontia and dental dysmorphologies with the use of post-natal administration of intravenous soluble recombinant EDA in X-linked HED dogs (Casal et al. 2007).

Although not yet demonstrated with the hypodontia phenotype, this line of research offers a promising future for hypodontia patients. Biological tooth substitutes and therapeutic use of recombinant proteins to correct the
pathological features of hypodontia could be possible. However, many obstacles must be overcome before such techniques become available as routine clinical treatment.

6.6 CONCLUSIONS

Recent research suggests that both environmental and genetic factors are involved in the aetiology of this condition, with the latter playing a more important role. It is also likely that specific hypodontia pathways have some effect on the function and psychosocial wellbeing of an individual, given the aesthetic, functional and financial burden for affected individuals.

This study aimed to: (1) investigate the association between non-syndromic hypodontia and genetic polymorphisms of candidate genes PAX9, MSX1, AXIN2 and EDA; and (2) examine the association between non-syndromic hypodontia and environmental factors, such as exposure to maternal smoking and alcohol during pregnancy.

Given the strengths and limitations of this study, and based on the data collected from the participants enrolled in this study, the following can be concluded:

1. Polymorphisms of the PAX9 and EDA genes may play a significant role in the aetiology of tooth agenesis.
2. The rs2520378 SNP of the EDA gene may protect against the development of hypodontia.
3. No significant associations between any of the studied SNPs on MSX1/AXIN2 genes and non-syndromic tooth agenesis were found.
4. There was an association between hypodontia and maternal smoking during pregnancy.
5. There is a biological gradient effect to this association, and this remained significant even after adjusting for confounders.

Therefore, although the exact molecular mechanisms involved in tooth agenesis remain unknown, the findings add to understanding of the genetic mechanisms underlying non-syndromic forms of hypodontia. As well as this, these findings establish a platform for future research in this area, and will add to understanding of the environmental influences on non-syndromic hypodontia.
7. REFERENCES


Ahn Y, Sanderson BW, Klein OD, Krumlauf R. 2010. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. Development. 137:3221 – 3231.


Olmsted MJ. 2011. Phenotype characterization and candidate genotyping of hypodontia in ectodermal dysplasia (ED) and non-syndromic groups [thesis]. [Chapel Hill (NC)]: University of North Carolina.


Dear Ms. Al-Ani,

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Institute/company: Faculty of Dentistry, University of Otago
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City: Dunedin
State/Territory: Country: New Zealand
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Book Year: 2007
Book Pages: 90 to 95
Book Chapter number: 5
Book Chapter title: Development of the Tooth and Its Supporting Tissues
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8.1.2 LITERATURE REVIEW SECTION – FIGURE 2.3

From: Professor Gabriella Galluccio (gabriella.galluccio@uniroma1.it)
Received: Tue 19/7/2016

Dear Dr. Al-Ani,

I am glad that you and Professor Farella have found useful what we reported in the article mentioned.

I had the opportunity also to discuss this request with all the other authors of the article and we have no problem in allowing the reproduction of the image, as indicated in your email.

It will be a pleasure in the future read your work.

Kindest regards

Gabriella Galluccio

Prof.Gabriella Galluccio
Direttore della Scuola di Specializzazione in Ortognatodonzia
Vice presidente del Corso di Laurea in Igiene Dentale B - Polo Molise
Corso di Laurea Magistrale in Odontoiatria e Protesi Dentaria
Sapienza Università di Roma
Viale Regina Elena 287/a,00161 Roma
T (+39) 06 49918157 & F(+39) 06 49976635

From: Azza Al-Ani (azza.al-ani@otago.ac.nz)
Sent: Sun 17/7/2016

Dear Dr Galluccio,

I am a final year Post-Graduate Orthodontic Student at the University of Otago, New Zealand, studying under the supervision of our Head of Department - Professor Mauro Farella. As part of our Clinical Doctorate degree, we must complete a thesis component. My project involves investigating the association between environmental and genetic factors with non-syndromic hypodontia.

The reason I am writing to you is to ask your permission to use Figure 1. from the paper published by your group in 2012: "Genetic basis of non-syndromic anomalies of human tooth number" in Archives of Oral Biology. I would be grateful if you allow me to do this, as I think that this is an informative figure. Of course, I will make sure to cite that this figure is from your paper, and that it was adapted from Brook (2009) etc.

Please do not hesitate to let me know if you have any queries regarding this matter, and I look forward to hearing back from you soon.

With best wishes,

Azza Al-Ani
BDS (Otago), DClinDent Candidate (Orthodontics, Otago)
From: Professor Alan Brook (alan.brook@adelaide.edu.au)

Sent: 8/8/2016

Dear Azza,

Great. I will write to Prof Bartold.

Best wishes,

Alan Brook
Professor, University of Adelaide

From: Azza Al-Ani (azza.al-ani@otago.ac.nz)

Sent: Sun 7/8/2016

Dear Prof,

I now have a copy of this paper, as I requested it from the library.


Thank you once again for your help.

With best wishes,

Azza
BDS (Otago), DClinDent Candidate (Orthodontics, Otago)

Dear Azza,

Thank you for contacting me and asking for permission to reproduce a figure.

May I suggest that rather than use the 2009 version of the figure you use the updated version which is Fig 6 in the Aust. Dent. J 2014 Special Supplement page 21…The Editor of the Aust. Dent. J. has already given approval in principle for reproducing figures from our papers and requires only an email from me for the journal’s records.

Kind regards

Alan
Professor, University of Adelaide
From: Azza Al-Ani (azza.al-ani@otago.ac.nz)

Sent: Thu 4/8/2016

Dear Professor Brook,

I am a final year Post-Graduate Orthodontic Student at the University of Otago, New Zealand, studying under the supervision of our Head of Department - Professor Mauro Farella. You may recall, we met briefly at the IADR ANZ Congress last year.

As part of our Clinical Doctorate degree, we must complete a thesis component. My project involves investigating the association between environmental and genetic factors with non-syndromic hypodontia.

I am interested in using Figure 2 (underlying scale of continuous variation determining tooth size and number) from the following article:


Once completed, my thesis will be available in print and electronic form for public access via the University of Otago institutional repository. The image of interest will of course be fully and correctly referenced if your permission is granted to include it in my thesis.

Thank you for your help with this, and I look forward to your reply.

Kind regards,

Azza Al-Ani

BDS (Otago), DClinDent Candidate (Orthodontics, Otago)
8.2 SNP SELECTION

8.2.1 PAX9

- Member of the paired box (PAX) family of transcription factors.
- These genes play critical roles during foetal development and cancer growth.
- Mice lacking this gene exhibit impaired development of organs, musculature and the skeleton, including absent and abnormally developed teeth, and neonatal lethality.
- Mutations in the human gene are associated with selective tooth agenesis.
- Located on chromosome 14

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References:


8.2.2 MSX1

- Encodes a member of the muscle segment homeobox gene family.
- Encoded protein functions as a transcriptional repressor during embryogenesis through interactions with components of the core transcription complex and other homeoproteins.
- May also have roles in limb-pattern formation, craniofacial development, particularly odontogenesis, and tumor growth inhibition.
- Mutations in this gene have been associated with nonsyndromic cleft lip with or without cleft palate, Witkop syndrome, Wolf-Hirschorn syndrome, and autosomal dominant hypodontia.
- Located on chromosome 4.

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8.2.3 AXIN2

- Involved in cell growth, proliferation and differentiation.
- AXIN2 is a negative regulator of the Wnt signalling pathway.
- Some evidence of the expression of AXIN2 in colorectal tissues leading to carcinomas.

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8.2.4 EDA

- The protein encoded by this gene is a type II membrane protein that can be cleaved by furin to produce a secreted form.
- Encoded protein, which belongs to the tumour necrosis factor family, may be involved in cell-cell signaling during the development of ectodermal organs.
- Defects in this gene are a cause of ectodermal dysplasia, anhidrotic, which is also known as X-linked hypohidrotic ectodermal dysplasia.
- Several transcript variants encoding many different isoforms have been found for this gene.
- Also known as: ED1; HED; EDA1; EDA2; HED1; ODT1; XHED; ECTD1; XLHED; ED1-A1; ED1-A2; EDA-A1; EDA-A2; STHAGX1
- Location on X-chromosome

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References:


**Several other papers discuss and study EDA, however, few specify the exact SNPs used.**
8.3 SNP GENOTYPING

8.3.1 QUALITY CONTROL OF THE SAMPLES

The samples were taken to the Merriman Laboratory at the University of Otago for storage, where DNA extraction of most of the samples was carried out. However, some samples were sent to the Agresearch Laboratory in Brisbane (Australia). Quality check procedures and genotyping were also undertaken at the Merriman Laboratory.

The steps for the curation of genotype were undertaken within Genome Studio. Genome Studio’s automatic clustering algorithms are reported to be accurate for ~99% of SNPs. The other ~1% need to be manually reviewed. The steps undertaken follow a guide that is consistent and logical, and involve a combination of those published by:


The gene chip used for genotyping was Human Genome Build 37, while Human Core Exome 24 v1.0 and v1.1 were used as support files to identify SNPs that were in the gene regions we were interested in.

8.3.2 GENOTYPE IMPUTATION

Genotype imputation is a statistical technique that is often used to increase the power and resolution of genetic association studies. Genotypes were imputed for the SNPs summarised in Appendix 8.2. The results of the imputations are outlined below, and from these, SNPs that were previously reported on in the literature were selected for the study.
<table>
<thead>
<tr>
<th>AXIN2</th>
<th>EDA</th>
<th>MSX1</th>
<th>PAX9</th>
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### 8.3.3 HARDY-WEINBERG EQUILIBRIUM

#### PAX9

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#### EDA

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</tr>
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<td></td>
<td>Control</td>
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<td></td>
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</tbody>
</table>

*Calculated for females only (Graffelman, 2016 #437)*
We would like to know a few details about you...

Date of Birth: 

Gender: 

- Male
- Female

Ethnicity: 

- New Zealand European
- Tongan
- Niuean
- Samoan
- Chinese
- Cook Island Maori
- Indian
- Other 

If applicable, who are your iwi?

If from the Cook Islands, what Island(s) are your parents from?

Thank You!
A University of Otago Research Study

Identifying the Genes involved in the Development of the Face and Teeth

Mother’s Questionnaire

Please try to answer all the questions, circling the “best” response. There are no right or wrong answers.

Please note that some of the questions may be sensitive; however, these questions are not intended to be offensive but rather to help us understand some of the biological processes involved in the development of the jaws and face. If you feel uncomfortable or unsure about a question, or you do not know the answer, you can leave it blank, or ask us for help or more information. All this information will be treated as confidential.
Before you start, we would like to know a few details about you...

<table>
<thead>
<tr>
<th>Date of Birth</th>
<th>/   /</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity</td>
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</tr>
<tr>
<td>New Zealand European</td>
<td>Tongan</td>
</tr>
<tr>
<td>Maori</td>
<td>Niuean</td>
</tr>
<tr>
<td>Samoan</td>
<td>Chinese</td>
</tr>
<tr>
<td>Cook Island Maori</td>
<td>Indian</td>
</tr>
<tr>
<td>Other</td>
<td></td>
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<tr>
<td>Address</td>
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</tr>
<tr>
<td>Current Occupation</td>
<td></td>
</tr>
<tr>
<td>Current Partner Occupation (Optional)</td>
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<tr>
<td>Phone Number</td>
<td></td>
</tr>
<tr>
<td>Email</td>
<td></td>
</tr>
</tbody>
</table>
And a few details about your family background...

Because this is a genetics study, we need to accurately understand the participant’s genetic origin - the best way to do this is from the ethnic origin of a participant’s biological father and mother’s parents (i.e. participant’s grandparents). If you do not know their origin, please indicate this with a question mark. Tick as many circles as you need within each box.
If applicable, who are your iwi?

_______________________________________________________________

If from the Cook Islands, what Island(s) are your parents from?

_______________________________________________________________
The next few questions are about your pregnancy and birth conditions in relation to the study participant...
The following questions relate to the pregnancy period and birth conditions of your child (study participant). Although this information relates to a long time ago, please try to answer these questions as accurately as possible.

**Exposure History (during pregnancy)**

<table>
<thead>
<tr>
<th>Question</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Did you drink caffeinated products? (e.g., coffee or energy drinks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Were you exposed to any form of x-rays?</td>
<td>Not At All</td>
<td>Dental</td>
</tr>
<tr>
<td>Did you smoke tobacco?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>If yes, how much? Cigarettes/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For how long during the pregnancy? Months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did anyone else in the house smoke (i.e., second hand smoking)?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Did you drink alcohol beverages? (e.g., wine, beer, spirits)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>If yes, how much? Glasses/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For how long during the pregnancy? Months</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pregnancy & Early Life**

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>How long was the duration of your pregnancy?</td>
<td>Weeks</td>
</tr>
<tr>
<td>Where was your child delivered?</td>
<td>Hospital</td>
</tr>
<tr>
<td>How long were you in labour with your child?</td>
<td>Hours</td>
</tr>
</tbody>
</table>

Was your child born early, late, or on time? (Gestational age)

<table>
<thead>
<tr>
<th></th>
<th>Early</th>
<th>Late</th>
<th>On Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

Can you remember the exact period? weeks
8.6 ETHICAL APPROVAL

Academic Services
Manager, Academic Committees, Mr Gary Witte

Dr J Antoun
Department of Oral Sciences
Faculty of Dentistry

20 August 2014

Dear Dr Antoun,

I am again writing to you concerning your proposal entitled “Finding the missing link for hypodontia”, Ethics Committee reference number H14/080.

Thank you for your letter dated 4th August 2014 addressing the issues raised by the Committee.

The Committee thanks you for your comment regarding Associate Professor Greg Jones’ concern over the study’s power to detect gene-environment interactions. Based on this concern, the Committee notes that you have revised the protocol to state ‘the primary focus of the study is to investigate the association between genes and the environment’, indicating that gene-environment interactions will only be analysed if the study power is deemed sufficient.

The Committee also appreciates the clarification given in respect of the data analysis and how the controls will be recruited for the study.

The Committee further notes the correction made to 7.10 of the application form and the rewording of the Parent Information Sheet and the development of an Information Sheet for ‘older children’.

On the basis of this response, I am pleased to confirm that the proposal now has full ethical approval to proceed.
The standard conditions of approval for all human research projects reviewed and approved by the Committee are the following:

Conduct the research project strictly in accordance with the research proposal submitted and granted ethics approval, including any amendments required to be made to the proposal by the Human Research Ethics Committee.

Inform the Human Research Ethics Committee immediately of anything which may warrant review of ethics approval of the research project, including: serious or unexpected adverse effects on participants, unforeseen events that might affect continued ethical acceptability of the project and a written report about these matters must be submitted to the Academic Committees Office by no later than the next working day after recognition of an adverse occurrence/event. Please note that in cases of adverse events an incident report should also be made to the Health and Safety Office:

http://www.otago.ac.nz/healthandsafety/index.html

Advise the Committee in writing as soon as practicable if the research project is discontinued.

Make no change to the project as approved in its entirety by the Committee, including any wording in any document approved as part of the project, without prior written approval of the Committee for any change. If you are applying for an amendment to your approved research, please email your request to the Academic Committees Office:

gary.witte@otago.ac.nz or jo.farrondediaz@otago.ac.nz

Approval is for up to three years from the date of this letter. If this project has not been completed within three years from the date of this letter, re-approval or an extension of approval must be requested. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing.

Yours sincerely,

[Signature]

Mr Gary Witte
Manager, Academic Committees
Tel: 479 8256 Email: gary.witte@otago.ac.nz

C.c. Professor R D Cannon Head Department of Oral Sciences
Finding the Missing Link for Hypodontia

INFORMATION SHEET FOR PARTICIPANTS or PARENTS / GUARDIANS

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

What is the Aim of the Project?

We are inviting you to take part in this study, which has been designed to help identify the genes that are involved in tooth agenesis – in other words, the failure of a tooth to form. Congenitally missing teeth is the most common type of dental anomaly to occur in humans and it represents a challenging clinical scenario for an orthodontist since it is difficult to achieve an ideal bite when teeth are missing.

Both the environment and our genes are believed to play an important role in the development of teeth. Understanding what genes are involved in this condition may potentially help improve our knowledge of the pathways involved in tooth development, and the identification of environmental risk factors may provide the best short-term opportunity for both personalised and population-level prevention. Other orthodontic and dental conditions that remain poorly understood may also benefit from this research.

Who are we looking for?

We are mainly looking for patients that have not yet started treatment with braces. In some cases, however, we may seek patients who have received or completed treatment to participate in the study. You will be invited to participate by your orthodontist if you meet the study’s selection requirement. We are searching for participants with one or more congenitally missing teeth.
(not including the wisdom teeth) – your orthodontist will be able to identify this during the clinical examination as well as from the routine jaw X-ray that is usually taken to help plan your orthodontic treatment.

Unfortunately, not everyone will be suitable for this study – patients with certain conditions (outlined below) may not be appropriate for our study as it may affect or distract us from what we are trying to find out. Your orthodontist will be able to check that you do not have any of these conditions, which include: missing teeth due to extraction; cleft lip and/or palate; and craniofacial syndromes.

How will this help people with orthodontic problems?

If you have a particular orthodontic problem, such as a missing tooth, it is unlikely that participation in this research will be of any direct benefit to you. Medical advancements typically take a long time and while we need your help to improve our understanding of tooth development, any meaningful breakthroughs may not happen for several years to come. In the future, it is hoped that better knowledge of the pathways involved in tooth development, along with the rapid advances in stem cell research, will facilitate the exciting prospect of tooth bioengineering.

As a personal “thank-you” for your time and effort in helping us with our study, we would like to offer you a small gift in the form of a Movie voucher.

What will you be asked to do?

Should you agree to take part in this project, you will be asked to provide a sample of your blood for genetic testing which will be collected on site by our research assistant who is a Registered Nurse, or alternatively a saliva sample. Blood samples are generally encouraged due to the higher quality of DNA that can be extracted from them. Good quality DNA will greatly help us find these genes involved in facial growth. Either form of these DNA collection methods (blood or saliva sample) will only be carried out once.

In addition to providing us with some personal information, such as age and ethnicity, participants’ mothers will also be kindly asked to answer a few questions that relate to their pregnancy with the participant. A qualified orthodontist may also carry out a simple check-up of your teeth and mouth. The questionnaire and clinical check-up should take approximately 20 minutes to complete.

Please be aware that you may decide not to take part in the project without any disadvantage to yourself of any kind.
What information will be collected and what will it be used for?

We will collect personal information such as gender, ethnicity and age. Any family history of congenitally missing teeth in your parents, siblings, grandparents, and other relatives may also be sought. In addition, we will collect clinical information (such as number of teeth present) as well as from the questionnaires that you will answer. This data will mainly help us during the analysis stages when we are trying to make sense of the results. If further information is required we may need to access your dental/orthodontic records – all of this information will stay strictly private.

DNA will be extracted from blood or saliva samples as described previously. By-products from this procedure are usually disposed of using medical waste contractors (please indicate on the consent form if you would prefer that a suitable Karakia be used for disposing of this genetic material). The samples, which may be used to study any related genes in the future, will be stored and tested in Dr Merriman’s laboratory at the University of Otago in Dunedin. Serum will also be stored for analysis of inflammatory markers that are related to the condition. All DNA samples will be stored in Dr Merriman’s laboratory.

The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve your anonymity. You will also be offered the opportunity to review the main findings of the study through the project’s website.

How will my data be stored and who will have access to it?

The data collected will be securely stored in such a way that only those mentioned below will be able to gain access to it. Data and DNA samples obtained as a result of the research will be retained for up to 10 years in secure storage. Any personal information held on the participants [such as contact details] may be destroyed at the completion of the research even though the data derived from the research will, in most cases, be kept for much longer or possibly indefinitely.

Only the research team will be able to access the above data and DNA samples. No other external source, commercial or non-commercial, will have access to any personal data or information.

Are there any risks?

Having a blood sample taken may hurt a little and some people may get a small bruise at the site where the blood is withdrawn. Although very rare, this site may become infected. Most people however have no problems with this routine procedure. If you have any bad experiences with giving blood samples,
please let the nurse know beforehand so she can accommodate for your special circumstances.

Can I change my mind and withdraw from the project?

Yes you can. You may withdraw from participation in the project at any time and without any disadvantage to yourself of any kind.

What if I have any Questions?

If you have any questions about our project, either now or in the future, please feel free to contact either:

Ms Azza Al-Ani / Mrs Cindy Mullens
Department of Oral Sciences
Faculty of Dentistry
University Tel: +64 3 479 7071
Email: azza.al-ani@otago.ac.nz

cindy.mullens @otago.ac.nz

Dr Joseph Antoun
Department of Oral Sciences
Faculty of Dentistry
University Tel: +64 3 479 7071
Email: joseph.antoun@otago.ac.nz

Climbing the Ladder, Together.

This study has been reviewed and approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph 03 479 8256). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.
Finding the **Missing Link for Hypodontia**

INFORMATION SHEET FOR CHILD PARTICIPANTS

Thank you for agreeing to consider helping us out. This sheet will explain to you what we are trying to do and hopefully help you decide whether or not to participate. In either case, we thank you for considering our request. Remember, there is nothing wrong with not participating if that’s what you prefer.

**What are we trying to do?**

Most people have 32 teeth. Just like most things though, some people are different in that they have one or more missing adult teeth. We are trying to find out what causes **these differences**. The number of missing adult teeth can sometimes make treatment harder and longer to treat and we want to try and improve things. One of the causes of missing teeth is in our genes - each person has a unique code (known as DNA), which plays a role in how many teeth each person will have. We are trying to find out which genes contribute to these differences in tooth numbers so we can predict things better and improve our orthodontic treatments. With your help, we may also be able to look at other dental problems that are controlled by our genes.

**Who are we looking for?**

We are looking for three kinds of volunteers: 1. People who have not started orthodontic treatment yet, 2. People who have braces, and 3.
People who are being followed up after they have had braces. Your orthodontist will let you know if you can help us with our study.

**How will this help people in the future?**

If you do have an interesting orthodontic problem (like a missing tooth/teeth), you probably won’t get much benefit from helping us out as anything we find will probably take a few years before can we make good use of it. But, hopefully, we will be able do simple tests in the future to predict whether someone has a missing tooth/teeth early on, and improve our current treatments. So by helping us, you will really be helping future children who will need orthodontic treatment.

**What will you be asked to do?**

We need two things from you – something to extract the DNA from, and some information from your mum about her time when she was pregnant with you.

Your DNA, which contains the genes we want to study, is found in either blood or saliva. We would like to take a very small sample of your blood to extract this DNA – this will involve you visiting a nurse or doctor who will do this for you. We prefer the DNA that we get from your blood as it helps us a lot more, but we can also collect some saliva instead if you really don’t want to give blood. Saliva samples involve spitting some of your saliva into a small tube – this can be done at your orthodontist's clinic. We will only need to collect your DNA once (either blood or saliva).

The second part involves Mum answering a few questions about some things when she was pregnant with you. These questionnaires should take about 20 minutes to complete.

**What will we do with your information?**

We will use your DNA sample and other information you have given us to study how the teeth fail to form. Your DNA sample will be stored and tested in Dr Merriman’s laboratory at the University of Otago in Dunedin (we may keep this information for up to 10 years).
We will write up the results from this study for our University work. The results may also be written up in journals and talked about at conferences, but your name will not be on anything written up about this study.

**Who will see my answers and other bits of information?**

Only the research team and the people we work with will look at the information you have kindly given to us.

**Can I change my mind and pull out from the project?**

Yes you can. You may pull out from participation in the project at any time and without any disadvantage to yourself of any kind.

**What if I have any Questions?**

If you have any questions about what we are doing, either now or in the future, please let us know:

**Azza Al-Ani / Cindy Mullens**  
University Tel: +64 3 479 7071  
Email: azza.al-ani@otago.ac.nz

**Joseph Antoun**  
University Tel: +64 3 479 7068  
Email: joseph.antoun@otago.ac.nz  
cindy.mullens@otago.ac.nz
Finding the **Missing Link for Hypodontia**

**CONSENT FORM FOR PARTICIPANTS**

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:

1. My participation in the project is entirely voluntary;
2. I am free to withdraw from the project at any time without any disadvantage;
3. At the conclusion of the project any raw data on which the results of the project depend will be retained in secure storage for at least five years;
4. The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve my anonymity.
5. At the end of the study, I consent to any remaining samples being disposed of using:
   - [ ] Standard disposal methods, OR;
   - [ ] Disposed with appropriate karakia,
6. I am happy at being contacted again in the future
   - [ ] No, I do not wish to be contacted again
   - [ ] Yes, but I understand that I do not have to participate in any further studies
7. In the unlikely event of exposure to blood products by staff, I consent to allow for testing of blood borne diseases to be undertaken.

I agree to take part in this project.

............................................  ............................................
(Signature of participant)          (Date)

This study has been approved by the University of Otago Human Ethics Committee, if you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator on 03 479 8256. Any issues that you raise will be treated with confidence and investigated and you will be informed of the outcome.
Finding the Missing Link for Hypodontia

CONSENT FORM FOR PARENTS/GUARDIANS

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:

1. My child’s participation in the project is entirely voluntary;

2. I am free to withdraw my child from the project at any time without any disadvantage;

3. My child will receive a small “thank-you” reward for their time and effort (movie or book voucher).

4. At the conclusion of the project any raw data on which the results of the project depend will be retained in secure storage for at least five years;

5. The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve my child’s anonymity.

6. At the end of the study, I consent to any remaining samples of my child being disposed of using:

   - Standard disposal methods, OR;
   - Disposed with appropriate karakia,

7. In the unlikely event of exposure to blood products by staff, I consent to allow for testing of blood borne diseases to be undertaken.

I agree for my child to take part in this project.

........................................................................................................... ....................................................
(Signature of parent/guardian) (Date)

...........................................................................................................
(Name of child)

This study has been approved by the University of Otago Human Ethics Committee, if you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator on 03 479 8256, Any issues that you raise will be treated with confidence and investigated and you will be informed of the outcome.
Finding the Missing Link for Hypodontia (Missing Teeth)

CONSENT FORM FOR CHILD PARTICIPANTS

I have been told about this study and understand what it is about. All my questions have been answered in a way that makes sense.

I know that:

1. Participation in this study is voluntary, which means that I do not have to take part if I don't want to and nothing will happen to me. I can also stop taking part at any time and don't have to give a reason.

2. Anytime I want to stop, that's okay.

3. If I don't want to answer some of the questions, that's fine.

4. If I have any worries or if I have any other questions, then I can talk about these with the research team.

5. The paper and computer file with my answers will only be seen by the research team and the people they work with and they will keep whatever I say private.

6. The research team will write up the results from this study for their University work. The results may also be written up in journals and talked about at conferences. My name will not be on anything written up about this study.

I agree to take part in the study.

Signed.....................................................................................
Date .......................................................................................
Wednesday, 23 July 2014.

Dr Joseph Antoun,
Faculty of Dentistry - Department of Oral Science,
DUNEDIN.

Tēnā koe Dr Joseph Antoun,

Finding the Missing Link for Hypodontia

The Ngāi Tahu Research Consultation Committee (the committee) met on Tuesday, 22 July 2014 to discuss your research proposition.

By way of introduction, this response from The Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum it states "Ngāi Tahu acknowledges that the consultation process outlined in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago". As such, this response is not "approval" or "mandate" for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee base consultation on that defined by Justice McGechan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon; adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

As this study involves human participants, the Committee strongly encourage that ethnicity data be collected as part of the research project. That is the questions on self-identified ethnicity and descent, these questions are contained in the latest census.

The Committee suggests researchers consider the Southern District Health Board’s Tīkaka Best Practice document, in particular patient engagement. The document also covers the collection, storage and disposal of blood and tissue samples. This document is available on the Southern District Health Board website.

The Committee suggests dissemination of the findings to relevant Māori health organisations, for example the National Māori Organisation for Dental Health, Oranga Niho and to Professor John Broughton, who is involved in Māori Dental Health, University of Otago.

The Ngāi Tahu Research Consultation Committee has membership from:
Te Rūnanga o Ėkākura Incorporated
Kāti Horataki Rūnanga ki Puketaki
Te Rūnanga o Māraki
We wish you every success in your research and the committee also requests a copy of the research findings.

This letter of suggestion, recommendation and advice is current for an 18 month period from Tuesday, 22 July 2014 to 2 January 2016.

Nīhaku noa, nā

Mark Brunton
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Research Division
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