

Genetic, Epigenetic, and Environmental Influences on Dentofacial Structures and Oral Health: Ongoing Studies of Australian Twins and Their Families

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The Craniofacial Biology Research Group in the School of Dentistry at The University of Adelaide is entering an exciting new phase of its studies of dental development and oral health in twins and their families. Studies of the teeth and faces of Australian twins have been continuing for nearly 30 years, with three major cohorts of twins recruited over that time, and currently we are working with twins aged 2 years old to adults. Cross-sectional data and records relating to teeth and faces of twins are available for around 300 pairs of teenage twins, as well as longitudinal data for 300 pairs of twins examined at three different stages of development, once with primary teeth, once at the mixed dentition stage, and then again when the permanent teeth had emerged. The third cohort of twins comprises over 600 pairs of twins recruited at around birth, together with other family members. The emphasis in this third group of twins has been to record the timing of emergence of the primary teeth and also to sample saliva and dental plaque to establish the timing of colonization of decay-forming bacteria in the mouth. Analyses have confirmed that genetic factors strongly influence variation in timing of primary tooth emergence. The research team is now beginning to carry out clinical examinations of the twins to see whether those who become colonized earlier with decay-forming bacteria develop dental decay at an earlier age. By making comparisons within and between monozygotic (MZ) and dizygotic (DZ) twin pairs and applying modern molecular approaches, we are now teasing out how genetic, epigenetic, and environmental factors interact to influence dental development and also oral health.

■ **Keywords:** teeth, faces, twins, dentistry, craniofacial development

Brief History

From the early 1920s, The University of Adelaide's School of Dentistry has conducted research on human growth and dental anthropology. Draper Campbell began by publishing a landmark thesis entitled 'Dentition and palate of the Australian Aboriginal' (Campbell, 1925). Murray Barrett and Tasman Brown followed on with a longitudinal growth study of Australian Aboriginals living in Yuen-dumu in the Northern Territory (Brown et al., 2011). The compilation of genealogical records for this population also enabled genetic analyses to be performed, with particular reference to tooth size (Townsend, 1980;

Townsend & Brown, 1978). These studies confirmed a relatively strong genetic influence on variation of dental crown size, and also showed that environmental factors played a role.

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In the early 1980s, a cohort of mainly teenage twins living in Adelaide, South Australia, was recruited to study teeth and faces with the aim to quantify the relative contributions of genetic and environmental influences to variation in dental and facial features. Many papers have been published based on the data generated from investigations involving this first cohort of twins. For example, it has been shown that there is a strong genetic contribution to human tooth size variation, with smaller common and unique environmental components of variance (Dempsey & Townsend, 2001). Plans are underway to follow up on these twins, now in adulthood.

In the 1990s, a collaboration was established with Professor Louise Brearley Messer at The University of Melbourne, and a second cohort of young twins was recruited for genetic analyses, focusing on dental and facial growth and development. This longitudinal study collected records at three key times of dental development: at the stage when all primary teeth were present (around 3–5 years), when children had mixed dentitions (around 8–10 years), and when all the permanent teeth were present except third molars (around 12–14 years).

A third cohort of twins was recruited Australia-wide between 2006 and 2010 for a study of tooth emergence and oral health. Key collaborations were established between investigators in Adelaide (led by Professor Townsend), Queensland (Professor Seow), and Western Australia (Professor Gotjamanos). The project is focused on clarifying the extent to which genetic factors contribute to variation in the timing and sequence of emergence of primary teeth. We are also determining the time at which mutans streptococci (MS), key organisms in the development of dental caries, first colonize the oral cavity of young twins. We are interested in determining whether initial colonization is linked to the emergence of primary teeth. An additional aim of this study is to clarify the pattern of transmission of MS within family members.

Recruitment Procedures

We have worked closely with the Australian Twin Registry and the Australian Multiple Birth Association to recruit twins for our studies. We have also actively recruited twin pairs for the third cohort from newspaper birth announcements, hospitals, and prenatal exercise classes. Retention rates throughout the studies have been high, with less than 10% attrition. We do not make any cash payments to participants, but we do provide dental products, gifts, and vouchers, as well as offering advice on oral health and reporting on any relevant findings from oral examinations. The following e-mail addresses are given as a first point of contact for anyone interested in our studies: toothfairy@adelaide.edu.au or michelle.bockmann@adelaide.edu.au.

Table 1 summarizes the sample sizes for the three cohorts of twins. At present, there are 1293 pairs of twins enrolled

TABLE 1

Twin Pairs Enrolled in Studies of Teeth and Faces of Australian Twins

	MZ ^a ♂	MZ ♀	DZ ^b ♂	DZ ♀	♂♀	Total*
Cohort 1	73	86	43	51	56	309
Cohort 2	84	79	50	54	60	327
Cohort 3	134	181	109	112	121	657
Total	291	346	202	217	237	1293

Family members enrolled						
	Mothers	Fathers	Brothers	Sisters	Other	Total
Cohort 1	65	52	19	35	7	178
Cohort 2	51	27	104	87	4	273
Cohort 3	657	645	350	155	—	1807
Total	773	724	473	277	11	2258

Note: *plus eight sets of triplets.

^aMZ = monozygotic.

^bDZ = dizygotic.

across three cohorts of twins. In addition, we have collected information for around 2,258 family members. Nearly all of the participants are of European ancestry.

Zygosity Determination

Zygosities of cohort 1 twins were confirmed by comparisons of a number of genetic markers in the blood (ABO, Rh, Fy, Jk, MNS) together with several serum enzyme polymorphisms and protein polymorphisms. Zygosities of twins in cohort 2 were confirmed by analysis of up to six highly variable genetic loci (FES, vWA31, F13A1, THO1, D21S11, FGA) on six different chromosomes, using DNA obtained from buccal cells. Zygosities for twins in cohort 3 have been confirmed by DNA analysis of 10 highly polymorphic genetic loci (D3S1358, vWA, FGA, AMEL, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) covering 10 chromosomes, from buccal swabs. We plan to distribute DNA kits to all participating members from each family enrolled in cohort 3 by the end of the year. OG-500 DNA kits will be used to collect saliva for further zygosity testing and genetic-based analysis.

Ethical Issues

Our investigations have the approval of the Committee on the Ethics of Human Experimentation, The University of Adelaide (Approval Nos. H-07-84A and H-78-2003) and all participants are informed volunteers.

Data Available for the Three Cohorts of Twins

The following types of records and information have been collected in our studies (Table 2).

Data Collection

For our most recent cohort, cohort 3, two questionnaires have been mailed to families, generally within the first

TABLE 2

Data Collection for the Three Cohorts of Twins

Cohort 1	Cohort 2	Cohort 3
<ul style="list-style-type: none"> • Oral examinations to record teeth present and to detect evidence of dental caries or other problems • Intraoral and extraoral photographs, standardized photographs of faces • Palm and fingerprints, laterality including hand, eye, and foot dominance • Detailed medical histories of the twins 	<ul style="list-style-type: none"> • Oral examinations to record teeth present and to detect evidence of dental caries or other problems • Intraoral and extraoral photographs, standardized photographs of faces • Palm and fingerprints, laterality including hand, eye, and foot dominance • Detailed medical histories of the twins 	<ul style="list-style-type: none"> • Oral examinations to record teeth present, information on soft tissue health (plaque, gingivitis) and the presence of caries, enamel defects, and wear on teeth • Intraoral and extraoral photographs
<ul style="list-style-type: none"> • Height and weight measurements • Alginate impressions of the upper and lower dental arches • Stereophotogrammetric records of facial features • Blood samples for DNA analysis and zygosity determination 	<ul style="list-style-type: none"> • Height and weight measurements • Alginate impressions of the upper and lower dental arches • Stereophotogrammetric records of facial features • Blood samples for DNA analysis and zygosity determination • Standardized facial profile photographs • Buccal swabs for DNA extraction, zygosity determination, and future linkage studies 	<ul style="list-style-type: none"> • Palm and fingerprints, laterality including hand, eye, and foot dominance • Detailed questionnaires on medical histories of the twins and their mothers, oral health histories, feeding habits • Height and weight measurements • Buccal swabs for DNA extraction, zygosity determination, and future linkage studies • Primary tooth emergence data

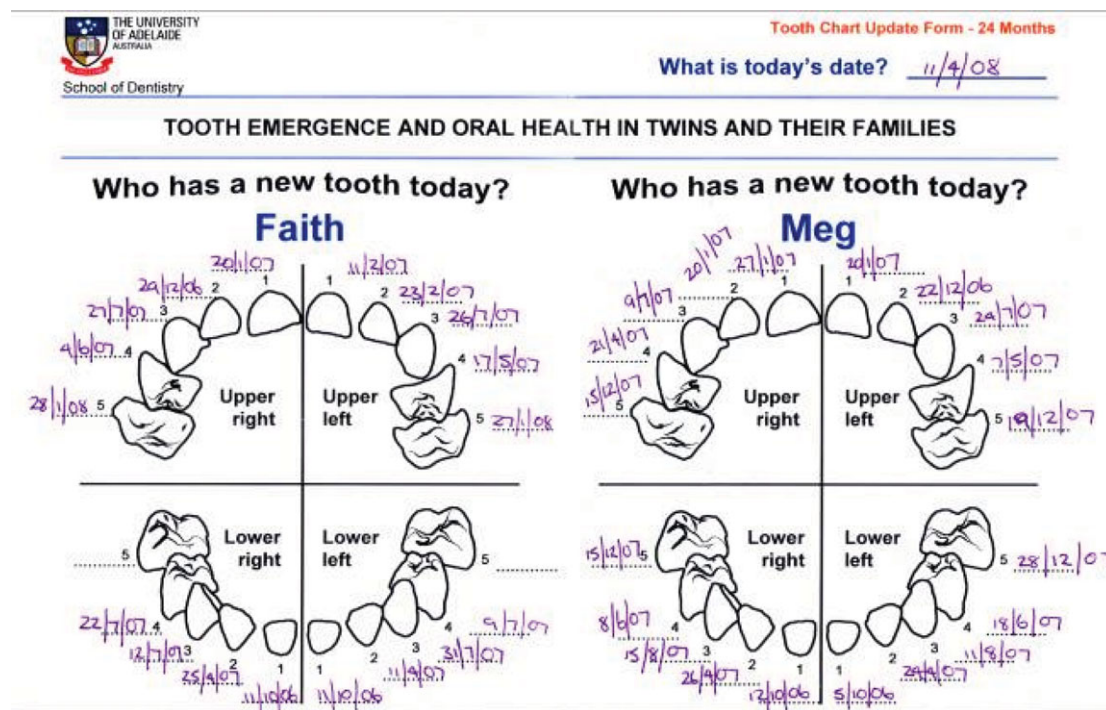


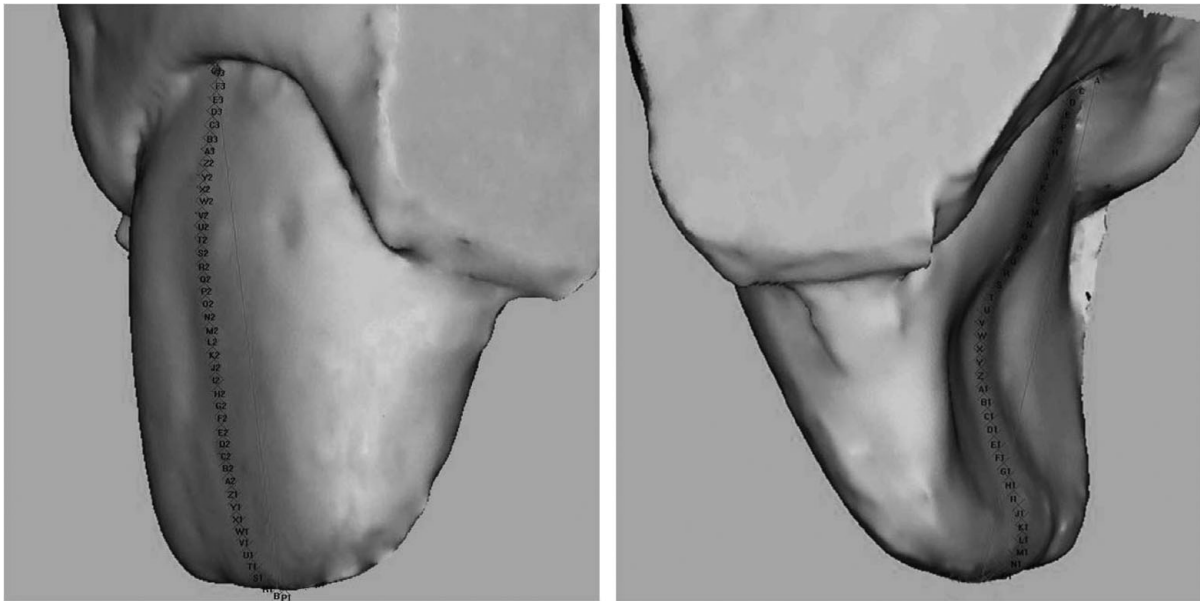
FIGURE 1

(Colour online) Example of a tooth emergence chart completed by parents.

2 years of the twins' lives. The questionnaires include items about pregnancy, delivery, birth weight and length, time in hospital, feeding habits, bottle usage, dental hygiene, fluoride intake, medication use, and parents' smoking habits and alcohol intake. A third questionnaire was also developed and mailed to families when the twins turned 4.5 years old, focusing more on the twins' oral and general health, medical history, as well as the oral health of the parents. Tooth chart diagrams were included in the question-

naire to indicate which teeth, if any, were decayed, missing (never erupted), filled, damaged from trauma or extracted.

Families also received tooth emergence and tooth exfoliation charts, to record the emergence and exfoliation of primary teeth. Both charts include a diagram of the primary dentition on which parents indicate the dates for which teeth have emerged/exfoliated (see Figure 1). Exfoliated teeth are collected by willing parents and sent to us for future analysis. Additionally, a 'Family Relationships' form has been

**FIGURE 2**

Examples of new dental phenotypes obtained using a 3D laser scanner.
 Note: Images generated by Dr Richard Smith and Professor Alan Brook.

sent to families to verify biological relationships between family members.

Colonization of MS in twins and other family members was determined by analysis of salivary/plaque samples, which were obtained by parents, or other family members, in their homes at 3-month intervals from when the twins were 3 months of age. The samples were posted to us and then plated onto selective media (TYS20BA) that support growth of MS, and incubated for 48 hours at 37°C in an atmosphere of 95% nitrogen and 5% carbon dioxide prior to scoring for presence or absence of MS. Identification of MS is currently based on its distinctive colonial morphology on the selective medium.

In 2011 and 2012, South Australian twins aged between 5 and 7 years were invited to participate in clinical sessions. A medical history was obtained and a clinical examination conducted on the twins, assessing soft tissues, plaque and gingivitis, presence and eruption status of primary and permanent teeth, and the presence and extent of caries, opacity, hypoplastic defects and wear. Twins were then tested for laterality (hand, eye, and foot dominance), and palm and fingerprints taken.

Data from cohorts 1 and 2 have been acquired by several methods, including directly measuring or scanning dental casts, and by digitizing landmarks on standardized photographs of individual teeth, dental arches, and facial photographs. Many different variables have been analyzed, including dental crown size, intercuspal distances, dental arch size and shape, occlusal variables such as overbite and overjet, and various facial dimensions. We have reported the results of replicability studies that have shown that errors of

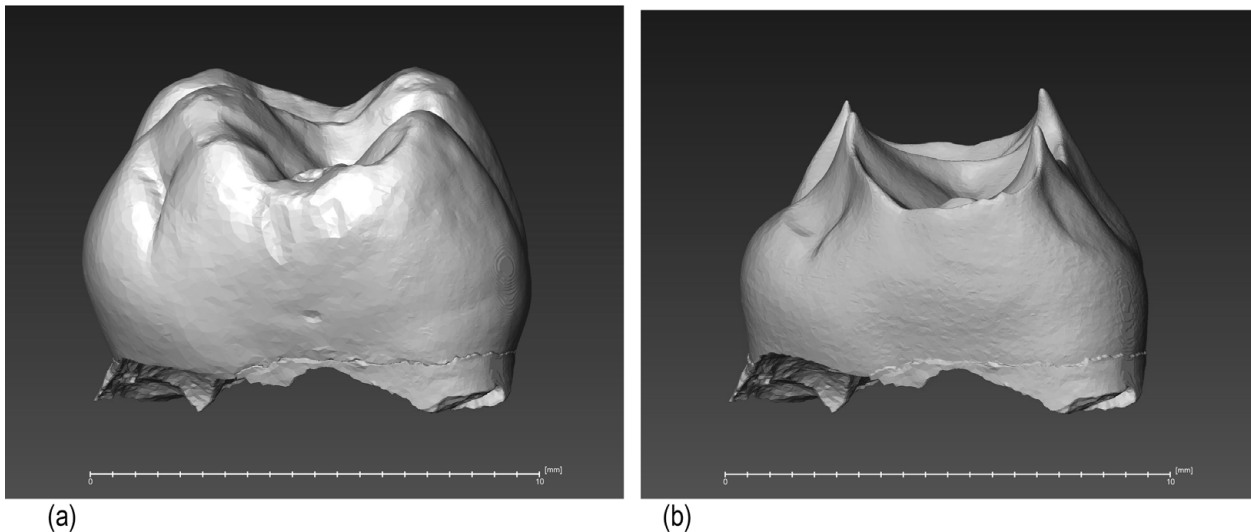
the methods are generally small and unlikely to bias results (Eguchi et al., 2004; Townsend et al., 2003).

We have employed the use of 2D and 3D imaging systems to measure human dental crown and arch size; 2D imaging uses a digital SLR camera while 3D imaging uses a 3D laser scanner. Both techniques have shown high levels of precision and accuracy, and will allow further development in the field of 'dental phenomics', where dental phenotypes can be quantified, in terms of contours, areas, and volumes, to provide detailed descriptions of the size and shape of teeth (Ashar et al., 2012; Townsend et al., 2012; see Figure 2).

Methods of Analysis

The equipment for stereophotogrammetric studies of facial morphology in our first cohort of twins consisted of matching left and right Hasselblad motor-driven cameras mounted on a rigid frame with an attached acrylic template fixed at a distance of 100 mm from the film plane of the cameras. We obtained five stereo views of the face for each subject — frontal, left and right profiles, and left and right half-profiles (Brown et al., 1987). For the second cohort of twins, we obtained standardized profile photographs of the faces of twins using a custom-built orthogonal photographic system in the School of Dental Science at The University of Melbourne (Tangchaitrong et al., 2000).

We have used the generalized structural equation modeling program, Mx, developed by Neale et al. (2002) to carry out genetic analyses of dental data in our twin sample (Dempsey et al., 1995). Heritability estimates (h^2), calculated as the ratio of additive genetic variation to total

**FIGURE 3**

Micro-CT images obtained of the same primary maxillary upper right second molar (mesial-proximal view). (a) with enamel cap, and (b) enamel cap removed showing dentine underneath.

Note: Images courtesy of Dr Jeremy Deverell and the Australian Microscopy & Microanalysis Research Facility at the South Australian Regional Facility (SARF), University of South Australia, a facility that is funded by the University, and State and Federal Governments.

phenotypic variation, have been calculated for several different dental and facial phenotypes (Hughes et al., 2000, 2001a, 2001b, 2012a, 2012b; Smith et al., 2009a; Townsend et al., 2009). More recently, we have been conducting developing multivariate models of dentofacial variation to account for the complex correlations between many of the phenotypes we have collected.

We have fitted orthogonal polynomial curves to dental arches and derived arch-shape coefficients. We are now expanding this approach using geometric morphometrics to describe variation in dental shape as well as size. When comparing dental morphologies between monozygotic (MZ) and dizygotic (DZ) twins, the datasets are not identical, making comparison of a particular region difficult. Recently, with our collaborators in Liverpool, we have used a minimized least-squares registration method to superimpose images of dental crowns in a pair of MZ twins. It appears that this diffusion-based registration method offers a more reliable approach to superimposing non-identical objects, such as dental crowns, than conventional best-fit methods (Smith et al., 2009b).

The dental and facial traits examined have been chosen to represent increasing levels of complexity within the dentition. We have considered factors influencing variation in individual teeth, including intercuspal distances and crown features such as Carabelli trait, which presents in various forms, ranging from a groove to a cusp on the mesiopalatal surface of permanent upper first molars or primary upper second molars (Townsend & Martin, 1992; Townsend et al., 2003). We have explored how genetic and environmental factors influence variation in the positioning of teeth within

and between the dental arches, as well as in selected facial features (Townsend et al., 2006). We have fitted orthogonal polynomial curves to dental arches and calculated heritability for arch shape coefficients (Hughes & Townsend, 2012a). We are now expanding this approach using geometric morphometrics to describe variation in dental shape as well as size. More recently, we have started to undertake 3D micro-CT of exfoliated primary teeth from cohorts 2 and 3 to explore the role of the genome in the development of the dental crown components (enamel and dentine; see Figure 3).

Apart from using the traditional twin approach, involving comparisons of MZ twin pairs and DZ twin pairs, we have also used the MZ co-twin design to investigate genetic and environmental influences on dental traits where one twin shows a feature and the other shows different expression or does not display the trait at all (Townsend & Brook, 2008). We have also looked at tooth size in twin pairs and found evidence for a possible male hormonal influence on dental development of females in utero (Ribeiro et al., 2012). Although we only have information on chorion type for a small proportion of our twin samples, we have been able to conduct some preliminary investigations of the relationship between chorion type, birth weight, and tooth size (Race et al., 2006).

Teeth, faces, and fingerprints are particularly suitable for studies of symmetry and asymmetry, and we have been particularly interested in studying the fascinating feature of mirror-imaging of dentition or different expression (Mihailidis et al., 2012, in press; Townsend & Brook, 2008; Townsend & Martin, 1992; Townsend et al., 1986).

Major Accomplishments

Since our earlier publication in the 2006 special issue of *Twin Research and Human Genetics* (Townsend et al., 2006), we have made significant progress in a number of key areas. Highlights include:

- Analysis of MS colonization in 151 MZ twin pairs from cohort 3 has shown that the mean age of colonization to be 12.7 ± 6.1 months; 25% of individuals were colonized prior to the emergence of the first tooth, and 21% of MZ twin pairs were discordant for colonization occurring before or after first tooth emergence (Bockmann et al., 2011). These findings suggest that environmental factors and/or epigenetic factors play a role in the colonization of MS in MZ twins.
- In a study of 98 Australian twins, 1–3 years old, strong genetic control in the variation in timing of emergence of the primary incisors was found, with a narrow-sense heritability estimated at 82–94% for males and 71–96% for females. There is also a small but significant contribution from the external environment (Bockmann et al., 2010; Hughes et al., 2007).
- Genetic modeling has shown that for a range of dental phenotypes the narrow-sense heritability range is 30–90%, suggesting an environmental contribution of 10–70%. Higgins et al. (2009) found a higher concordance in hypocone (the distopalatal cusp of upper molars) expression in MZ twins compared with DZ twins and a high narrow-sense heritability estimate. Hasegawa et al. (2007) compared mandibular first molars (M1) and second molars (M2) between Mongolians (belonging to the Khalkha-Mongol grouping) and Caucasians (Northern European ancestry). It was suggested that both genetic and environmental influences might contribute to the differences observed in buccolingual dimension of M1 between Mongolians and Caucasians, whereas the mesiodistal and buccolingual diameters of M2 may be under more environmental influence since M2 develop later and over a longer period of time (Hasegawa et al., 2007).
- Preliminary analyses from the clinical examinations carried out in 2011 show some prevalence of caries and filled teeth (<25%) and particularly high levels of enamel opacities (around 75%). Hypoplastic defects were less common in around 12–19% of the sample. There tended to be a symmetrical pattern of distribution intra-twins; however, different patterns of distribution were found between twins, for pairs concordant for enamel defects.
- Together with other colleagues involved in twin research, we contributed laterality records to a large study of 54,270 Australian and Dutch twins and their non-twin siblings to characterize the heritability of handedness (Medland et al., 2009).

- Woodroffe et al. (2010) investigated tooth emergence in 216 Australian twin pairs. The first primary tooth emerged at around 8.6 months and the last primary tooth at around 27.9 months, with an order of central incisor, lateral incisor, first molar, canine, and second molar. Primary anterior teeth appear to be emerging later when compared with studies conducted in 1984 and 2003, but the sequence of emergence has remained unchanged.
- Studies of 2- to 4-year-old twins and singletons were conducted to investigate enamel hypoplasia (EH) and dental erosion (DE). Twins showed a higher prevalence of EH compared with singletons, and greater concordance was noted between MZ pairs than DZ pairs. It was concluded that both genetic and environmental factors contribute to variation of EH, with environmental factors likely exerting a greater influence. The prevalence of DE was found to be similar between twins and singletons, suggesting genetic factors contribute very little to the development of DE (Taji et al., 2010, 2011).
- A study was conducted on 409 Australian twin pairs primarily to explore the influence of chorion type on the timing of first primary tooth emergence, and also to explore the relationships between birth weight, gestational age, and birth length. Low birth weight and low gestational age were associated with significant delays in the timing of the first primary tooth emergence regardless of chorion type. However, monozygotic monochorionic (MZMC) male co-twin pairs discordant for birth weight were more likely to be discordant for timing of first primary tooth emergence (McConnell et al., 2011). This was explored further with an analysis of 217 twin pairs, which found extreme prematurity or very low birth weight led to significant delays in the emergence of the first primary tooth. A significantly later emergence time of 10.7 months occurred in extremely premature infants (gestation <30 weeks) compared an emergence time of 7.0 months with full-term infants (gestation ≥ 37 weeks). Very low birth weight babies (<1500 g) also showed a significant delay in tooth emergence at 10.1 months compared with 7.9 months for normal birth weight babies (>2500 g). Investigation into feeding practices found the mean first primary tooth emergence times to be 8.0 and 8.6 months for breast-fed and bottle-fed babies, respectively, which was not statistically significant (Chan et al., 2012). Further exploration of these relationships is planned with colleagues, Professor Dorret Boomsma and her research group at VU University, Amsterdam.

Discussion and Future Directions

Our cohorts of twins, with a focus on teeth and jaws, are the largest of their type in the world, spanning over 30 years of data collection. We are now moving into an exciting new time with more emphasis on molecular aspects.

- Epigenetic studies: We have obtained whole-genome microarray methylation profiles for a subset of MZ twins from cohort 2, discordant for missing/extra teeth. These data are in the early stages of analysis, but there is a suggestion that MZ twins discordant for number of teeth show a greater degree of discordance in methylation status at a whole-genome level, than corresponding concordant pairs (Williams et al., 2012). The next phase of the analysis will examine methylation status at loci known to be associated with dental development.
- Oral microbiome work: Currently, there is little genetic information available about the timing and acquisition of the developing oral microbiota during childhood, and whether species other than *Streptococcus mutans* in the infant oral microbial community are indicative of an individual's oral health status in early childhood. Furthermore, at a genetic level, we are unaware of what factors shape the composition of the developing oral microbiota, which is interacting with both a changing environment (e.g., diet and medications) and host-related factors. In collaboration with Dr Christina Adler and Professor Neil Hunter at the University of Sydney, we aim to determine if the composition of the oral microbial community in infancy is predictive of the clinical presentation of oral health or caries in early childhood through the use of in-depth genetic analysis. In particular, we plan to examine the V3 to V6 (nucleotide position 341–1046) hypervariable region of the 16S rRNA gene in oral swabs from selected pairs from cohort 3.
- Phenotyping: We plan to continue intensive dental phenotyping from all available cohorts. For cohorts 1 and 2, we will apply modern 2D and 3D imaging systems enabling biologically meaningful, dental phenotypes to be quantified in order to provide detailed descriptions of the size and shape of teeth. We propose that developments in the field of 'dental phenomics', with linking of the data generated to large-scale genome sequencing approaches, should enable us to further unravel the mysteries of how genetic, environmental, and epigenetic factors interact to produce the extensive range of morphological variations evident within the human dentition and face (Townsend et al., 2012).
- Following the establishment of an International Collaborating Centre in Orofacial Genetics and Development at the University of Liverpool in 2007, collaborative initiatives between our group and the Director of the Centre, Professor Alan Brook, have developed substantially. The Centre is now structured as an 'international network' and Professor Brook holds professorial appointments at Queen Mary, University of London, UK, and also in the School of Dentistry at The University of Adelaide, South Australia. One exciting aspect of this recently expanded multidisciplinary collaboration involves using complex adaptive systems to model the complexity of

the developing dentition (Brook & Brook O'Donnell, 2012)

- We intend to extend our studies of dermatoglyphic patterns, including assessment of the nature and extent of asymmetrical expression, to determine whether there are similar genetic factors operating on the development of fingerprints and dental crown morphologies. Given that both of these structures develop from epithelial–mesenchymal interactions, we are particularly interested in determining whether there are associations evident in the patterns of expression between these two phenotypes.

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