Confirmation Report

Linking general health and oral health – A study of twins

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1.1 Abstract

Through toothache, infection and tooth loss, dental diseases have a major impact on the health and well being of children. Dental caries and hypomineralised second primary molars (HSPM) are two common diseases of childhood. In both, prenatal and early life environment are thought to be important influences but remain incompletely understood. The aim of this project is to investigate the contribution of genetics, shared and non-shared environment to the variation in risk of dental caries and HSPM, and to identify specific shared and non-shared factors from the pre- and perinatal period that are likely to be associated with disease.

1.2 Null hypotheses

1. MZ and DZ twins are equally correlated for risk of dental caries.
2. MZ and DZ twins are equally correlated for risk of HSPM.
3. Pre- and perinatal shared and non-shared factors are not associated with dental caries at age 6.
4. Pre- and perinatal shared and non-shared factors are not associated with HSPM.

2 Literature Review

Introduction

Oral health is an integral part of general health. Yet despite advances in prevention and management, a significant number of children suffer pain, infection and hospitalisation due to dental conditions. Poor childhood oral health is a legacy for a lifetime of poor oral health and its implications extend from the child to the family to the wider community (1). The worldwide cost of dental diseases has been estimated at US$442 billion annually (2). Furthermore, there is increasing evidence for an interaction between oral disease, including periodontal disease and dental caries, and general health, in particular non-communicable diseases (NCDs) such as cardiovascular disease and diabetes (3). This interplay is particularly important in light of the significant morbidity and mortality associated with NCDs which, in 2008, accounted for approximately 63% of global deaths (4).

Dental caries and molar incisor hypomineralisation (MIH) are two of the most common dental conditions in childhood. In both, the risk is likely to be determined very early in life, perhaps *in utero*. Understanding the role of pre- and perinatal influences on these conditions may help reduce the burden of oral diseases by improving preventive approaches.

Part 1: Dental Caries

Epidemiology and impact

The decline in caries experience reported up to the turn of the century appear to be reversing, particularly in young children, such that almost half of six year old Australians today have dental caries (5). Furthermore, in common with other chronic diseases, dental caries is not experienced equally across the population with one in ten Australian children having more than 10 carious teeth (4 - 5 times the national average) (6).

Treatment of dental disease costs the Australian economy $6.27 billion per year (7). Over 26,000 Australians under 15 years of age are admitted to hospital for the treatment of dental caries annually, making it the highest cause of acute, preventable hospitalisations (8). By twelve years of age, a third of Australian children have experienced toothache, leading to difficulty eating, playing and attending school (9). Due to these factors, dental caries has been associated with poor nutrition, growth and development and impaired children's and caregivers' quality of life (10). Childhood dental caries experience is the strongest indicator of future poor oral health (11). Preventing caries in children is likely to have a significant impact on long-term oral health outcomes in adulthood (11).

Pathogenesis

The pathogenesis of dental caries starts within the plaque biofilm, a structured community of host-derived glycoproteins, bacteria and their by-products which forms on the surface of teeth within a matter of hours (12). With time and adequate substrate, the plaque bacteria produce organic acids, mostly lactic, formic and acetic, that favour the survival of aciduric species (13). These organic acids in turn, lead to a desaturation of the plaque fluid with respect to tooth mineral, triggering the diffusion of minerals from the adjacent tooth surface, creating a net loss of mineral (14).

However, this process can reverse rapidly to result in net remineralisation once the acidity is neutralised or saturation re-established with added calcium and phosphate. Mechanical disturbance of the biofilm through toothbrushing and acid buffering by salivary proteins, bicarbonate and other agents can allow the uptake of minerals such as calcium, fluoride and phosphate back into the tooth surface once super-saturation with respect to tooth mineral is re-established.

Therefore, dental caries is a dynamic process and the clinical manifestations such as enamel surface cavitation result when the balance between pathological factors that lead to demineralisation and carious lesion development overwhelm the protective factors that favour remineralisation, resulting in substantial mineral loss (Figure 1) (15).

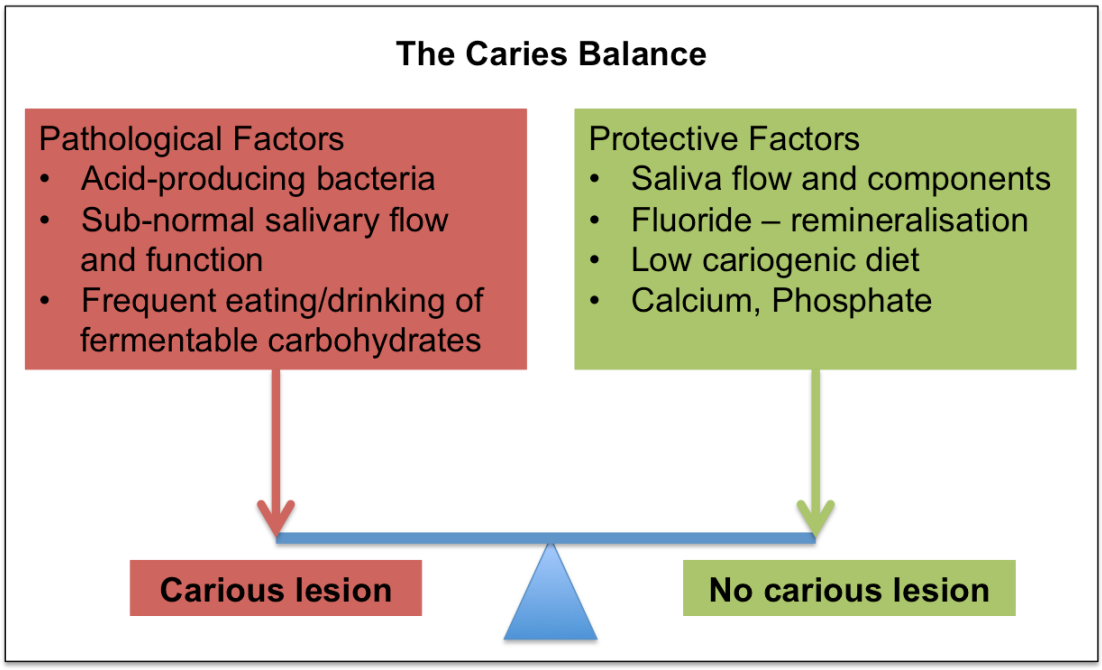


Figure 1: The balance between protective factors and pathological factors. (15)

Clinical presentation

The early carious lesion presents clinically as a white spot lesion of enamel, due to subsurface demineralisation that alters the translucency (refractive index) of the enamel (14). By the time such a lesion is evident, considerable mineral loss may have already occurred in the body of the lesion and the outer layer of dentine and pulp may also be involved (16). Nevertheless, through mechanical removal of the plaque biofilm, intrinsic protective factors and use of preventive agents, remineralisation is still possible at this point due to a relatively intact outer enamel surface layer. However, once cavitation has occurred, and the outer layer has broken down, the dentine may have become extensively involved (17). Direct colonisation of the dentine by bacteria, in the presence of adequate substrate will result in further acid production, lesion progression and pulpal involvement. At this stage, sealing and/or restoration of the cavitated lesion to eliminate access to substrate and restore structural integrity of the tooth is necessary.

Aetiological factors

Despite its high prevalence and associated health, psychosocial and economic burdens, the cause of dental caries remains incompletely understood. It is well

recognised that carious lesions result from the interaction of acidogenic and acidoduric bacteria, substrate (fermentable carbohydrates) and a susceptible tooth surface (Figure 2) (17). However the aetiology is far more complex, including a range of genetic and environmental factors, starting in the prenatal and early childhood period

Although dental caries is no longer considered a transmissible and infective disease, microbial species are essential in the pathogenesis of dental caries (18).

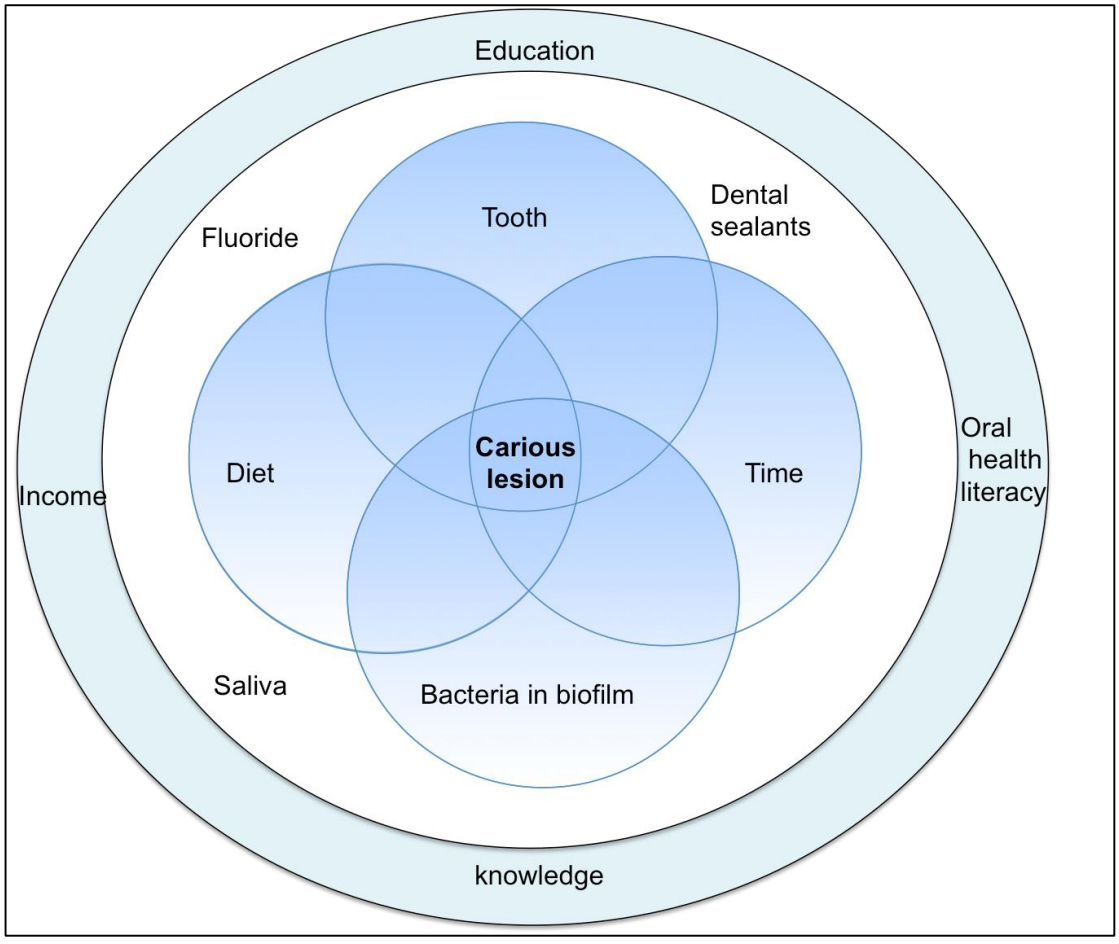


Figure 2: Factors involved in the development of carious lesions (17).

Bacteria

Encompassing several different habitats, the oral cavity is host to one of the most complex collections of microbiota in the human body. The salivary microbiome has been investigated in a number of studies, although perhaps not to the same degree as the plaque biofilm (19-24). A better understanding of the salivary microbiome and in

particular, how it is influenced by environmental and genetic factors may not only contribute to the evolving concepts of the host-microbiome interplay in health and disease but open exciting prospects in the field of salivary diagnostics.

Oral Microbiome in health

The human body has as many bacteria as it does human cells (25). In most cases, these microbial populations exist in a symbiotic, or mutually beneficial relationship with the host (26). While the host provides a nutritious habitat, microbial species perform a range of important developmental and metabolic roles for the host (27). The gut microbiota is essential for the development of lymphoid follicles in the small intestine and production of IgA (28). In the oral cavity, commensal bacteria that have a better affinity for the habitats in the oral cavity prevent colonisation by potentially pathologic exogenous species by preferentially colonising the host (27).

Although the host microbiome appears to be primarily determined by the host species, studies of the oral microbiota in unrelated healthy individuals seem to indicate that a "core microbiome", shared between all healthy individuals exists (29). Based on preliminary studies, this appears to be unaffected by geographic factors (22). Nevertheless, each individual appears to be have a unique microbiome, to such a degree that it has been suggested as an alternative means of identification (20).

The oral cavity is unique in that is contains both shedding mucosal surfaces as well as non-shedding tooth surfaces. The resident microbiota at different sites within the oral cavity may be grouped into three broad groups that share similar composition: (1) the buccal mucosa, keratinised gingiva and hard palate; (2) the saliva, tonsils, tongue, and throat; and finally (3) supragingival and subgingival plaque (23).

Although microbial invasion of the amniotic sac has generally been associated with obstetric complications such as pre-term birth, there is emerging evidence that even in healthy pregnancies, an individual's exposure to microbes may occur *in utero* (30). While the developing foetus is likely to be protected and therefore have limited interactions with microbial agents, either directly, or indirectly through maternal

immune responses, these may be sufficient to result in significant changes in the microbiome in infancy which in turn may increase susceptibility to disease (31). Obesity in pregnancy has been (in sheep models) associated with a genetically driven increase in placental inflammatory mediators, which subsequently have been suggested to program the developing foetus to an increased risk of immune, inflammatory and metabolic diseases (32). However, most of the evidence for this prenatal modulation of an individual's microbiome and future disease risk comes from studies of the gut microbiome and much is experimental. No similar studies have been conducted on the oral microbiome or the implications of any such changes on oral or other diseases.

Despite the paucity of evidence regarding prenatal factors, there is consensus that the mode of delivery appears to dictate the microbiome in newborns. Vaginally-delivered babies have a microbiome reflecting the mother's vaginal microbiome whereas caesarian delivery results in a newborn microbiome more closely resembling that of the skin (33). In both situations, the microbiome is similar across a range of body habitats.

There is a paucity of evidence regarding the development of the oral microbiome after birth. By six months of age, the infant microbiome has been reported to be highly diverse and no longer reflective of the mother (19). The factors that influence this change in the microbiome are not yet understood. A study of the gut microbiota has shown that a significant proportion is inherited (34). In contrast, the only similar study of the oral microbiome, a longitudinal study of twins and unrelated adopted siblings from 12 to 22 years of age reported little genetic influence (24). However, although the authors concluded that shared environment is the most important factor determining the composition of the oral microbiome, they could not show any significant association with environmental factors such as diet, weight and sex. Physiologic changes that occur throughout life, such as eruption of teeth, maturation of the immune system and endocrine changes during puberty and pregnancy also influence the oral microbiome, which adapts to these new physiological challenges (26).

Dysbiosis, a shift in the microbiome leading to disease can occur in response to number of factors, including the use of broad-spectrum antibiotic therapy or frequent intake of fermentable carbohydrates (27). This concept underlies a significant paradigm shift in the understanding of the pathogenesis of many diseases, including oral diseases such as dental caries and periodontitis that were previously thought to be caused by a limited number of specific pathogens (18).

Oral microbiome and dental caries

Due to the limitations of culture-based laboratory techniques, dental caries has traditionally been considered an infectious disease, with Streptocccus mutans being the principal pathogen (35). However, with rapid developments in culture-independent techniques, which enable a much broader and deeper analysis of the microbiome, evidence is emerging to indicate that the oral microbiome and its role in disease is much more complex (36). Streptococcus mutans has been detected even prior to eruption of teeth and in oral environments without clinically detectable dental caries, suggestive of it being an endogenous species rather than an exogenous infectious agent (18). A host of other species, including Actinomyces, Bifidobcaterium, Proprionibacterium and non-mutans Streptococci have been associated with dental caries, supporting a relatively complex bacterial contribution (37). In addition, dental caries has been shown to occur or progress without significantly elevated levels of Streptococcus mutans (38, 39) (37-40). As such, there is unlikely to be specific caries determining species. Instead, dental caries is likely to occur when dysbiosis results in a biofilm that facilitates demineralization (41, 42).

The nature of the dysbiosis appears to favour more acidoduric and acidogenic bacteria. Some studies have suggested that as plaque matures and the plaque microbiome shifts to become more acidic, the resulting conditions may lead to a reduction in bacterial diversity (38, 43). However, a number of other studies suggest greater diversity with dental caries (21, 39, 44). Larger, longitudinal studies assessing site specific changes in plaque microbiome are needed to further clarify nature of the dysbiosis that leads to dental caries.

Modifying the host, microbial and environmental factors that drive the dysbiosis that leads to dental caries is therefore more likely to result in effective preventive techniques than elimination of specific species. Such an approach necessitates a better understanding of these factors, as there is still little known.

Given the close links between microbiome and immunity, genetically-controlled host factors, such as inflammation and immune response may well modify the transition from symbiosis to dysbiosis (26). Dental caries has been shown to have some degree of heritability and a number of genes have been implicated in susceptibility to the disease (45). Genetically controlled host immune factors may at least in part, explain this association (46). Exposure to prenatal maternal factors such as obesity and smoking have been associated with subsequent increased caries-risk in childhood. In addition to shared lifestyles and confounding by social factors, intra-uterine programming and modulation of host immunity could be a plausible biological explanation for this association, albeit one, which needs further evidence (47-49).

Although most investigations into microbial changes in dental caries and indeed other oral diseases focus on identifying the taxonomy, microbial factors are more likely to be related to function (28). Not only do different strains within species exhibit a range of metabolic activities but these are likely to be influenced by the environment within the biolfilm. There is ample evidence that indicates the consumption of high and persistent levels of sugar influences the metabolic activity of the oral microbiome (50). However, other factors can also influence bacterial function. For example, the authors of an *in vivo* study suggested that the preventive effect of fluoride is in part due to inhibition of the bacterial enolase, a key component of the Embden Meyerhof Parnas pathway used by bacteria to produce acid from dietary carbohydrates (51). In addition, environmental factors like oxygen saturation and pH are major drivers of "phenotypic plasticity", altered gene expression within bacterial species that favour more cariogenic phenotypes (52).

The frequent intake of fermentable carbohydrates is a well-researched factor driving the dysbiosis that results in dental caries (53, 54). In addition, other lifestyle and dietary factors such as use of broad-spectrum antibiotics, breast feeding, exposure to pets and siblings and preventive therapies, including the use of probiotics particularly

early in life when the microbiome is considered to be at its most malleable, may also be important. Although there is now a considerable body of evidence about how these may affect the gut microbiome, in particular by influencing diversity of microbial species, there is little information regarding the oral microbiome. A better understanding of how these environmental factors affect the composition and function of the oral microbiome may lead to improved preventive modalities.

Susceptible tooth surface

Dental carious lesions may occur on either the coronal or root surfaces of teeth, which by nature of their structure and ultrastructure are inherently susceptible to demineralisation. Although dental caries is generally reported as a single measure of disease activity in an individual, susceptibility can vary for different teeth and tooth surfaces. A study of a high caries-risk adult population identified five clusters of tooth surfaces with similar occurrence of carious lesions. Although the clusters were generated from surface level data, surfaces within each cluster were generally contiguous and symmetrical (55). A similar study in children found that the dentition can be organised in to a hierarchical structure with the buccal pits and occlusal fissures of the first permanent molar teeth being most likely be affected by carious lesions and the mandibular anterior teeth and canines being least susceptible (56). Importantly, the authors reported that the effect of risk factors and preventive mechanisms are likely to be different between different clusters but similar within each cluster. The apparent difference in susceptibility between clusters of surfaces, in particular pit and fissures versus smooth surfaces, however, has been attributed to the differential influence of risk factors (57).

Developmental defects of enamel although not essential, may favour demineralisation and cavitation in the dynamic balance in the caries process and is discussed further under ‘risk factors’ (58).

Substrate

Evidence

Sugar intake has been conclusively shown to be an important aetiological factor in the dental caries process (59). Free sugars include monosaccharides and disaccharides that are added during food and beverage preparation, as well as those naturally present in honey, syrups, fruit juices and fruit juice concentrates (60). Fermentable carbohydrates refer to free sugars, glucose polymers, fermentable oligosaccharides, and highly refined starches (61). The pioneering Vipelhom study that first demonstrated this relationship by comparing caries increment in residents of a Swedish mental institute who were fed different dietary sugar regimens may be less relevant in the post-fluoride era (62, 63). Nevertheless, in a recent systematic review of the effect of sugar on caries, 42 of 55 eligible studies reported a positive association between sugar intake and dental caries prevalence and incidence. Double-blind randomised controlled trials are difficult to conduct because the use of sugar-free sweeteners, which would be needed to ensure the participants are blinded, may themselves have effects on the caries process (61). In addition, measuring and classifying sugar intake is challenging. However, the Turku studies comparing the caries incidence in a control group with sucrose intake, and two intervention groups where sucrose was replaced entirely by xylitol and fructose found markedly higher caries levels in the sucrose and fructose groups (64). There is also strong support for causality from population studies, including Japan and Iraq where the sugar supply was restricted following World War II and UN restrictions respectively, leading to drastic reductions in caries prevalence (65, 66). When the access to sugar eventually increased in Japan, so too did the caries prevalence (65).

Mechanisms for the aetiological role of sugar in dental caries

Most of the evidence underpinning the mechanisms by which dietary factors lead to the development of carious lesion is based on *in vitro* studies or un-blinded *in situ* studies where study participants wear a removable prosthesis with enamel blocks that are subjected to sugar exposures extra-orally at various time intervals. Simple sugars such as sucrose or its constituent monosaccharides glucose and fructose are highly cariogenic, primarily because they are readily metabolised by plaque bacteria with acid as a metabolite (67, 68). This not only results in loss of mineral from the tooth

surface due to loss of super-saturation with respect to tooth mineral, but also precipitates a progressive shift of the plaque microbiome from one in a symbiotic relationship with the host to dysbiosis favouring growth of aciduric and acidogenic species (53). Critically, sucrose has added cariogenicity because it triggers the production of intra- and extracellular polysaccharides such as glucans (IPS and EPS) (69). EPS help bacterial adherence onto the tooth surface and support the structure and organisation of the plaque biofilm in dental caries, allowing the movement of substrate into deeper parts of the biofilm to maintain a highly acidic environment adjacent to the tooth structure (50). IPS are stored as secondary nutrient sources when exogenous (dietary) substrate is not available. Plaque biofilms formed in the presence of sucrose have also been reported to have lower concentrations of calcium, inorganic phosphate and fluoride, inhibiting remineralisation potential and increasing the presence of under-saturation with respect to tooth mineral (50). The underlying mechanism for this association is still unclear although a study showing reduced levels of calcium-binding proteins in plaque formed in the presence of sucrose suggests that a depletion of calcium reservoirs may play a role (70).

Non-sucrose substrate

Although plaque bacteria are able to metabolise both simple mono- and disaccharides like, fructose, glucose and sucrose as well as processed starches, the evidence for a causal role for starch in dental caries is not yet definitive (71). Starches describe a broad category of food types that contain varying numbers of a number of different complex carbohydrates within starch granules. Food preparation and various other factors can influence the release of starch molecules from their granules, making them available for breakdown and metabolism by host and bacterial factors (71). Controversially, Campain et al reported that intake of starch, in the form of cereal, corn chips, French fries, pretzels and bread was more frequently associated with increased caries-risk than sugar in a group of low-caries risk adolescents. In particular, there was a higher risk of developing carious lesions with a high-starch low-sugar diet compared to groups with either a high-sugar low-starch or medium-sugar medium starch diet (72). A plausible explanation for the potential role of starch in dental caries is the reduced oral clearance of starchy foods, which results in a slow, albeit prolonged, release of sucrose as the complex carbohydrates are metabolised

(73, 74). Alternatively, other factors such as frequency or timing of intake may explain the relationship, as high-starch low sugar foods are more likely to be consumed as snacks. Evidence for this comes from a longitudinal study of a relatively high socioeconomic status American cohort that found increased caries incidence with consumption of processed starches (potato chips etc) at snack times, but not during meal times (75). The aetiological role of dietary starches is likely to depend on various factors, including the type of food, the degree of processing, frequency and timing of consumption but further studies are needed to clarify the relative importance of these factors.

Although lactose is a disaccharide and therefore potentially metabolised by plaque bacteria to produce inorganic acids, dairy products such as bovine milk and cheese appear to be protective of dental caries (76). A study of rats given a high sugar diet showed that the group supplemented with bovine milk had reduced numbers of carious lesions compared to a control group (77). As bacterial profiles between the two groups and amount of sugar intake were similar, the protective effect was attributed to another factor within the milk, most likely the presence of relatively high concentrations of calcium and phosphate. A more recent *in vitro* study comparing soy extract and bovine milk revealed much smaller fall in pH in the bovine milk group, which the authors attributed to the acid buffering capacity of the milk and presence of increased bioavailability of calcium in and presence of milk proteins (78).

The influence of fluoride on the relationship between sugar and dental caries

Several systematic reviews have suggested that the aetiological role of diet is less critical in the post-water fluoridation era (62, 79). However, as argued by Sheiham et al., such a modification seems to reflect on the effectiveness of fluoride as a preventive modality rather than the relationship between dental caries and sugar, because the underlying need for sugar in the dental caries process is still applicable (61).

Time

Time is an important underlying factor in the development of dental caries. Firstly, the shift in plaque biofilm from symbiosis to dysbiosis takes time although the rate at

which this occurs, as previously discussed is likely to dependant on several environmental factors, especially exposure to substrate (17). In addition, as teeth are constantly fluctuating between demineralisation and remineralisation, in order for carious lesions to develop, there has to be a sustained period of net demineralisation (15).

Risk factors for dental caries

Beyond the four main aetiological factors that directly contribute to the development of carious lesions (e.g. bacteria, substrate, tooth surface and time), a range of environmental factors is likely to influence disease experience (Figure 2) (17). A recent systematic review found a total of 106 risk factors have been associated with dental caries in young children, with varying levels of strength of evidence (80). A detailed assessment of the literature pertaining to each of these factors is beyond the scope of this review. Nevertheless it would be remiss not to acknowledge the considerable wealth of evidence supporting the importance of socio-economic status, dietary and oral hygiene behaviours and fluoride exposure in caries risk (1, 61, 81).

The association between some factors, such as salivary flow or exposure to fluoride, can be readily explained by the current understanding of dental caries as a balance between remineralisation and demineralisation. However, the mechanism for other factors is more indirect and speculative, particularly for prenatal and early life factors (82).

The identification of risk factors prior to disease onset is essential for prevention. As dental caries can occur very early in life, from the prenatal and perinatal period and early childhood are therefore particularly important. Although few studies have investigated a connection, epigenetic changes induced by early life factors may also provide a plausible explanation for the association of such factors with dental caries (83). The role of prenatal and early childhood factors have generally only been studied in young populations, however, based on the theory of developmental origins of health and disease, they may be important even later in life (84).

Vitamin D

Through its role in calcium homeostasis, vitamin D is essential for the formation of the dental tissues and mineralisation of enamel (85). Vitamin D synthesis occurs in the skin as a result of exposure to sunlight (UVB) (86). In addition, vitamin D may be ingested through a limited number of foods, including certain fish, eggs and fortified margarine and dairy products, or taken as supplements. Vitamin D circulates in the body in the form of 25-hydroxy Vitamin D, and is converted into its active form, 1,25-dihyroxyvitamin D in the kidney. Once activated, vitamin D increases renal and intestinal absorption of calcium and also affects the level of parathyroid hormone and phosphate. Lack of vitamin D can lead to reduced levels of calcium and phosphate, which in turn leads to abnormal mineralisation of calcified structures. A systematic review and meta-analysis of controlled clinical trials found a 47% reduction in caries-risk following dietary vitamin D supplementation or UV radiation (87). However, the authors acknowledged a reasonable risk of bias due to questionable methodology in 22 of the 24 studies, all which had been conducted more than 60 years earlier. The applicability of these findings for today's populations is also questionable. Indeed, very few recent studies have assessed the relationship between dental caries and vitamin D levels. A case control study comparing vitamin D levels in children with severe early childhood caries and a control group from the community found reduced vitamin D levels in the cases (88).

While low vitamin D has been reported as a risk factor for childhood caries, a prospective study linking prenatal maternal vitamin D levels with dental caries in the first year of life was the first to study this association during pregnancy (48). As the study controlled for the effect of enamel hypoplasia, socio-economic status and feeding patterns amongst other potential confounders, the association between vitamin D and dental caries is likely to involve a unique mechanism. However, the representativeness of the cohort is questionable as 90 per cent were Canadian Aboriginal children at increased risk of vitamin D deficiency. A more recent Japanese prospective cohort study reported a similar association, finding children in the lowest quartile of maternal vitamin D intake at increased risk of developing caries. However the study had several major methodological limitations, including calculating vitamin D based on a diet questionnaire without accounting for sun exposure (89). The most representative study, evaluating data from the NHANES

2005-2006 failed to find an association between vitamin D deficiency and dental caries (90). The cross-sectional design of the study has limitations because caries experience may be the result of previous vitamin D deficiency which would be undetected by measuring vitamin D at one time point only.

The studies do not elaborate on possible mechanisms other than due to reduced availability of calcium and phosphate, which may increase the susceptibility of the tooth surface to caries. However, given recent studies indicating that vitamin D has a range of functions in several tissues, including in immune modulation, an alternative pathway involving increased susceptibility to infection cannot be discounted (91). Further longitudinal studies assessing the relationship between vitamin D and dental caries are needed to clarify whether an association exists and whether treatment of vitamin D deficiency can reduce caries-risk.

Maternal smoking

Although studying the effect of parental smoking is difficult to measure due to the role of confounders such as socio-economic status, dietary patterns and oral hygiene behaviours, there are a number of relatively robust observational studies supporting an association. A large cross-sectional study using NHANES data from 1988-1994 reported that elevated nicotine levels were associated with decayed surfaces (adjusted odds ratio 1.8, 95% confidence interval 1.2 – 2.7), after adjusting for several potential confounders (92). These findings are supported by evidence from the UK National Diet and Nutrition Survey (1995) that found maternal smoking was associated with increased odds of dental caries after stratifying for social class (93). However, a recent systematic review found that although possible links between second-hand smoke and dental caries exist, further longitudinal studies are needed (94).

There are a limited number of studies that have shown that an association between smoking and dental caries may exist from as early as the prenatal period. A longitudinal register-based Swedish study reported that smoking during pregnancy was associated with an increase in approximal caries increment during adolescence (47). Further, a cross-sectional study based on NHANES (1999 – 2002) data reported an association between maternal smoking in pregnancy and dental caries (95).

However, these results contrast those of a Japanese study that found the association between maternal smoking during pregnancy and dental caries in childhood became insignificant when adjusted for postnatal second hand smoke exposure) (96). Although the authors acknowledge this effect could be due to the low number of women who ceased smoking after pregnancy, this study reflects the difficulty in differentiating the role of factors in the prenatal and post-natal period.

Although still speculative, parental smoking may lead to developmental defects by interfering with tooth mineralisation or facilitate dysbiosis, either directly, or by altering immune-related factors (94). However, due to the strong links between smoking and social disadvantage, a major modifier of health outcomes, and caries-risk behaviours, a causative link may not exist. Although many studies do account for some of these factors, bias is impossible to avoid completely without randomised control trials. Presuming a possible causal link, mechanisms for the association between prenatal maternal smoking and dental caries include a direct influence on immunity in the developing foetus, or abnormal tooth mineralisation. A recent animal study found hard tissue aberrations in mandibular molars of rat foetuses after maternal exposure to second hand smoke, including delayed development and reduced thickness, volume, density and hardness (97). The authors suggested that nicotine may interfere with ameloblast proliferation and differentiation, or reduce blood supply to the developing tooth.

Maternal obesity

Two cohort studies from Scandinavia reported obesity during pregnancy was associated with higher caries rates in the children (47, 49). As both studies adjusted for several confounders including socio-economic status and smoking, the authors suggested that these observations were due to biological influences on the developing foetus, or transfer of dietary habits.

Maternal obesity has been reported to be associated with higher risks of childhood obesity. Although shared dietary and lifestyle preferences may be a major factor in this association, epigenetics may also lead to ‘foetal programming’ due to influences

in the intra-uterine environment (98). The links between obesity and dental caries are controversial, despite a number of studies showing an association (99).

Maternal illness and medication use

There are few studies comprehensively investigating the relationship between maternal illness during pregnancy and dental caries. A study of maternal illness, including the pre-pregnancy period, failed to find any association. However, as illness was classified under three broad categories and illnesses from the prenatal and post-natal periods combined, possible associations with illness in the prenatal period may have not been detected (49). A Saudi Arabian study investigating pre-eclampsia and dental caries in childhood also failed to find a significant association. However the measurement of dental caries was not with a standardised index and the study is likely to have been underpowered to find an association if one existed (100).

A recent study has reported that antibiotic use during pregnancy may reduce the risk of childhood caries (adjusted odds ratio 0.30, 95% confidence interval 0.07-1.27) (101). Although the confidence interval suggests weak evidence for such an association, the authors speculated that antibiotic use may lead to reduced vertical transmission of bacteria. However, given that the microbial dysbiosis that leads to dental caries is relatively complex and cariogenic bacteria are commensal, such a simplistic explanation seems unlikely. Further studies on the role of maternal illness and antibiotic use during pregnancy in dental caries are needed.

Low birth weight and prematurity

Low birth weight is associated with a number of co-morbidities. Low birth weight is defined as birth weight of less than 2500g (102). Further sub-categories within this definition include very low birth weight (less than 1500g) and extremely low birth weight (less than 1000g). In Australia, the rates of low birth weight have remained relatively stable over the last decade, at approximately 6% of all live births (103).

Although dental caries has been suggested as another co-morbidity associated with low birth weight, a number of studies have reported an association in the opposite direction, with lower rates of dental caries in children born with low or very low birth

weight, or no association at all (104, 105). A recent systematic review found that although there was no evidence of an association between dental caries and low birth weight, a potential association could not be discounted because of the lack of good quality evidence (106).

Evaluating the association between low birth weight and dental caries is challenging because low birth weight is an indicator of overall social disadvantage, itself a strong predictor of dental caries (106). Biological links through developmental defects of enamel, which are strongly associated with low birth weight and prematurity are plausible explanations for an association with dental caries (104, 107). Low birth weight children enrolled in longitudinal cohort studies are likely to have better access to dental care, and may therefore be at reduced risk of dental caries.

Low birth weight is either due to prematurity (less than 37 weeks of gestation) or intra-uterine growth restriction (IUGR) (108). As these have different risk factors and effects on the child, their effects on dental caries may also be different. A recent study evaluating prematurity as well as two different measures IUGR revealed associations in opposite directions (109). Whereas pre-term birth was associated with higher levels of dental caries, IUGR was found to be protective. The authors explain these findings by citing changes in oral bacterial due to eruption delay and increased use of antibiotics amongst children with IUGR. However, the latter is controversial, as antibiotic use also reported to be associated with early childhood caries (110).

Mode of delivery

Delivery mode appears to affect various facets of the human microbiome and as such, has led to suggestions that it may be associated with dental caries experience (33). A Danish cross-sectional study reported that children who were delivered vaginally were more likely to foster health-related bacteria in saliva (111). A prospective study found caesarean delivery was associated with earlier colonisation by S. mutans, and reduced diversity in bacterial strains (112). Given the important role played by oral bacteria in dental caries, the mechanisms for these associations need further investigation. However, speculation about the impact of such factors on microbiome is often simplistic, returning to the now discounted theories regarding S. mutans as the cause of dental caries. Indeed, the two studies evaluating the association with dental

caries are in disagreement with the above. A cross-sectional Thai study of 3-5 year old children found higher levels of dental caries and S. mutans in children born vaginally and a Danish study failed to find any association between dental caries and delivery mode (113, 114). Further studies investigating the impact of mode of delivery on the oral microbiome may clarify the process that leads to dysbiosis that results in carious lesions, but at present there is insufficient evidence for a direct association.

Part 2: Molar Incisor Hypomineralisation and Hypomineralised Second Primary Molars

Developmental defects of enamel (DDE) include qualitative or quantitative lesions that result from disruptions during amelogenesis which is the highly specialised, but sensitive process of enamel formation. (85) Molar incisor hypomineralisation (MIH) describes DDE characterised by demarcated, qualitative defects of enamel of systemic origin affecting one or more first permanent molars (FPMs) with or without incisor involvement (Figure 3) (115). Hypomineralised Second Primary Molars (HSPM) is a relatively new term, used to describe similar lesions in the second molars of the primary dentition (116). Although the occurrence of HSPM is associated with MIH, each can occur without the other (117). As MIH was defined almost a decade earlier, it has been much more widely investigated than HSPM. However, as the two conditions are similar in most aspects, findings from many studies of MIH are thought to apply equally to HSPM.

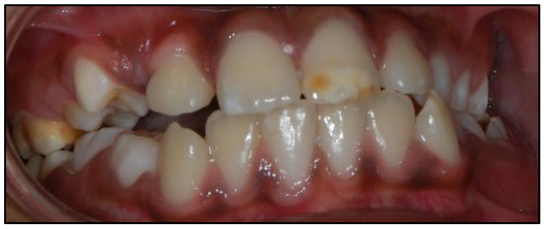


Figure 3: MIH affecting right first permanent molar and left central

Sound enamel is comprised mostly of an inorganic crystal matrix of impure hydroxyapatite organised into prisms that run from the dentino-enamel junction to the surface. The enamel in MIH has been found to be abnormal in terms of mineral quantity, with reduction up to 45 per cent reported, and quality, due to incorporation of carbonate (118). The protein content in hypomineralised enamel is significantly higher than in normal enamel and comprises of exogenous proteins such as serum albumin and antitrypsin (119, 120). In addition, ultra-structural abnormalities such as poor crystal organisation and surface defects have been reported (121). The culmination of these abnormalities results in enamel with increased porosity, decreased hardness and lower modulus of elasticity (118, 121, 122).

Clinical Presentation

As a result of its structure, sound enamel is translucent to visible light. Defective mineralisation in MIH increases enamel porosity, changing the refraction of light such that an opacity is visible (123). The presence of proteins may contribute to the discolouration in MIH opacities (119). The most common and mildest clinical presentation of MIH are demarcated white, creamy opacities (Figure 3a). Yellow or brown opacities are also frequently encountered and indicate a greater degree of hypomineralisation. (124).

Upon loading with masticatory forces, MIH/HSPM affected enamel is susceptible to breakdown, resulting in irregularly shaped and often expansive Post-Eruptive Breakdown (PEB) cavities (124). MIH and HSPM affected teeth may also develop carious lesions in atypical patterns, involving smooth surfaces and cusp tips as opposed to the pit and fissure and approximal surfaces that are affected in typical dental caries (122).

Lesions tend to occur on the occlusal, buccal and lingual/palatal surfaces of molars although approximal surfaces have reported to be involved in a minority of cases (122, 124, 125). In permanent incisors, the lesions tend to occur on buccal surfaces (124-126). In all MIH teeth, the cervical regions of teeth are unaffected (122). The lesions may be as small at 2mm in diameter or so extensive that multiple tooth surfaces are involved but generally tend to be demarcated from the adjacent sound enamel. A key feature of MIH and HSPM is that teeth forming at the same time may be affected in markedly different degrees (127).

The clinical presentation of MIH and HSPM is a function of several factors, including the severity of the hypomineralisation, masticatory forces, exposure to remineralising agents and the time since eruption (125). Post-eruptive breakdown is thought to usually occur soon after eruption, which is approximately 2-3 years of age for the second primary molars and 6 years for the FPMs. Reflecting severity of hypomineralisation, darker lesions are at greater risk of breakdown.

Unlike other DDE which are either generalised, affecting the entire dentition or localised, affecting a tooth or teeth in one part of the mouth, MIH/HSPM, by definition, affects only specific teeth, albeit in most cases, to varying degrees (128). However there is evidence that similar lesions may affect other teeth in the dentition (129, 130). Nevertheless, lesions on teeth other than the second primary molars, FPMs and permanent incisors are currently not included in the within the scope of the definition.

From the limited number of studies available, the degree of hypomineralisation appears to be more severe in HSPM when compared to MIH, with higher rates of post-eruptive breakdown and atypical caries reported (117, 131). However, this may be a reflection of the earlier eruption of the second primary molars, meaning a longer time exposed to the challenges within the oral environment.

MIH and HSPM Judgement criteria

The ideal time to assess a tooth for the presence of MIH/HSPM is soon after eruption, prior to the various influences within the oral cavity. However, due to the variability in eruption times, it maybe necessary to diagnose MIH months, even years after the tooth has erupted. As the presentation of MIH and HSPM can vary with time, the judgement criteria for identifying MIH/HSPM accommodates these influences (132, 133). In addition to demarcated opacities, atypical restorations on the FPMs are indicative of MIH, as treatment for MIH and HSPM where PEB has occurred involves multiple surfaces of the tooth resulting in patterns not typical of traditional dental caries. Due to rapid destruction of tooth structure, extractions are more common among children affected by MIH than those without the condition. Where the loss of a FPM(s) is not consistent with the condition of the remaining dentition, and there is evidence of MIH on other incisors or molars, the missing tooth is considered to have been affected by MIH/HSPM.

Prevalence and impact

The prevalence of MIH in Australia is reported to be 22%, which is slightly higher than the median values reported worldwide, ranging from 2 - 40% (134, 135). The

prevalence of HSPM in a cohort of inner-suburban pre-schoolers in Melbourne, Australia has recently been determined to be 16%, which is considerably higher than the 4 – 9% reported in the limited number of international studies available (116, 131, 136, 137).

There is emerging evidence of the major, and long-lasting impact of MIH and HSPM on children. MIH can lead to toothache, dental caries, infection and tooth loss. Teeth with MIH have been reported to be hypersensitive, particularly to cold stimuli but even spontaneously in severe cases (115). MIH affected teeth often do not respond effectively to local anaesthesia, making restorative treatment difficult, especially in children who are already anxious due to hypersensitive teeth (138). In addition, MIH affected teeth have poor treatment outcomes, often necessitating multiple attempts at restoration (139). The combination of hypersensitive teeth that do not respond to anaesthesia but require frequent treatment is likely to account for the higher rates of dental anxiety and behaviour management problems in children with MIH (139). As a result of behaviour management problems and the complex nature of treatment, expensive advanced behaviour management techniques such as sedation and general anaesthesia are often needed (140). The burden of MIH extends into adulthood, with MIH-affected eighteen-year-olds found to have a significantly higher number of extracted and filled teeth than unaffected controls and persistent behavioural issues (141).

Aesthetics

Although incisors are generally less severely affected than molars in MIH, unaesthetic demarcated opacities can be the source of considerable concern to those affected. A recent British study found that children with opacities on anterior teeth, most frequently MIH, were more likely to suffer from unpleasant comments from peers, impacting on confidence and willingness to smile (138, 142). These add to the psycohological strain caused by difficult dental treatments from a young age.

Orthodontic complications

Early or delayed extraction of a FPM can result in major orthodontic complications, including tilting of the adjacent teeth, super-eruption of opposing teeth and spacing of

the dentition, possibly including shift of the midline (143). Although favourable timing of extractions may alleviate some of these concerns, the severity of problems may mean that early extractions are needed (143). In addition, issues regarding access to care, cost, lack of clinician experience and behaviour management problems may result in extractions being delayed. Early loss of primary teeth, as may occur in HSPM, has also been shown to lead to orthodontic complications later in life. The need for specialist orthodontic treatment adds to the treatment burden of MIH.

Dental Caries

A number of studies have found that MIH and HSPM are risk factors for development of carious lesions, consistent with other DDE (144, 145). Defective enamel in DDE is likely to be less resistant to demineralisation, increasing susceptibility to caries progression. In addition, rough margins resulting from post-eruptive breakdown of enamel and a reluctance of children to brush hypersensitive teeth may further contribute to caries progression in MIH. The study of the association between MIH/HSPM and dental caries has not been straightforward, complicated by difficulty defining the difference between caries and MIH, especially where restorative treatment has been undertaken. Most studies reporting an association between MIH have compared the number of decayed, missing or filled teeth (122, 125, 145). Elfrink et al reported that HSPM explains the higher rates of caries affecting second primary molars compared to the first primary molars (144). A German study, one of few that failed to find an association between MIH and dental caries excluded any FPM with atypical restorations from their caries index (146). However given that MIH may also give rise to atypical caries, this may have led to an under-estimation of dental caries in MIH affected teeth. MIH and HSPM are likely to have a relatively bigger impact on dental caries and treatment need in low caries populations where improvements in preventive modalities, from water fluoridation to improved oral hygiene and dental care and the influence of traditional risk factors has been reduced.

Aetiology

Amelogenesis occurs in stages, commencing with an initial secretory phase involving the laying down of the organic matrix by specialised cells, the ameloblasts. Subsequent phases of mineralisation and maturation occur during which much of the

organic protein is replaced by impure hydroxyapatite mineral resulting in the highly mineralised tissue that is tooth enamel. The timing of an insult therefore determines the nature of the enamel defect. Disruptions during the secretory phase result in quantitative defects, also known as hypoplasia. Disruptions during the mineralisation and maturation phases result in qualitative defects such as MIH. As mineralisation of the FPM occurs between the third trimester of pregnancy and three years of age, investigations on the aetiology of MIH have focused on prenatal or early childhood illness or exposure to environmental pollutants. The permanent incisors and the primary second molars also mineralise during this time, thereby explaining their involvement in MIH/HSPM. The more protracted duration of mineralisation of the FPMs may account for the more severe involvement of these teeth in comparison to both the permanent incisors and second primary molars. Alternatively, as the FPM is a bigger tooth than the second primary molar, the greater thickness of enamel may lend it self to a more pronounced clinical presentation.

A combination of *in vitro* studies of the structure and composition of MIH/HSPM affected teeth and epidemiological studies suggest that the aetiology relates to a prenatal or early childhood illness. However, the search for a cause has been hampered by a lack of prospective studies and a specific cause(es) has not yet been determined.

Observational studies of MIH/HSPM aetiological factors

A number of systematic reviews have been conducted on the aetiology of MIH, one of which is part of this PhD and is the basis of this section of the literature review. Two systematic reviews conducted in 2010 and 2009 found a lack of good quality evidence and recommended further studies with improved study design and standardised examination and diagnostic protocols (147, 148). A number of studies have since been published, contributing significantly to the evidence regarding MIH aetiology.

Prenatal exposures

A Greek case-control study found that 8.6 per cent of mothers of children with MIH had medical problems during the prenatal period, mostly fever or medication use. In

contrast, none of the mothers in the control group reported any problems (149). However, few other studies have been able to find a link between MIH and maternal health and illness during pregnancy. Three prospective studies have found no association between maternal smoking during pregnancy and MIH (150-152). Ten studies have assessed the association between maternal illness during pregnancy and MIH, one of which (151) was a case-control study that used data collected prospectively. A Brazilian retrospective study that stratified results into rural and urban areas found an increased odds ratio for MIH in children whose mothers had health problems during pregnancy, but only in rural areas (153). Another retrospective cohort study investigated specific pregnancy related condition and found a positive association between MIH and hypotension-related anaemia and maternal stress (154). The authors noted that due to recent wars and sanctions, their sample may have unique social conditions that limit application to other countries. A large retrospective cohort study from Turkey found that once adjusted for confounding, the association between MIH and maternal illness in the last trimester of pregnancy was not significant (155). There was considerable variability in the terms used to describe maternal illness during pregnancy between studies, with some specific to pregnancy-related disease and others reporting more broadly in terms of maternal illness. An association between maternal medication-use has been investigated, and discounted in a number of mostly retrospective studies.

Prenatal factors may however be more important in the aetiology of HSPM. As only three reports (two of which were from the same study) investigating the aetiology of HSPM were available, there is considerably less evidence regarding its aetiology than for MIH (156-158). However, as both studies have relatively low risk of bias and adjust for confounding and as one is prospective in nature, the findings are relatively robust. The Dutch studies conducted by Elfrink et al indicated that while maternal antibiotic use during pregnancy is unlikely to be associated with HSPM, maternal alcohol intake may be (156, 157). Ghanim et al reported that prenatal health events were present in approximately one quarter of children affected by HSPM, compared to only 4.1 per cent of unaffected children (158). Children with both HSPM and MIH were excluded from the study, which raises questions regarding how to investigate the aetiology of the two conditions which maybe different but also related.

Perinatal exposures

A questionnaire based retrospective study found that complicated vaginal delivery and caesarean delivery were both associated with increased odds of MIH (159). Ghanim et al also identified perinatal events as important, with children with MIH more frequently having low birth weight and neonatal complications such as respiratory distress, intubation and hypocalcaemia (154). In addition, with birth information obtained from a registry, Brogardh-Roth et al found that per 100g increase in birth weight, the odds of MIH reduced by 0.96 fold (95% CI 0.92-0.99) (160). The study's main aim was to investigate the association between MIH and pre-term birth by comparing a group of pre-term babies and a group of term children from the community. The MIH prevalence in the pre-term group, at 38 per cent was double that of the full-term children. These findings were also supported by the findings of the Turkish retrospective study by Sonmez et al, which found increased odds of MIH among premature children, even after adjusting for confounding (155).

Perinatal events have been reported to be important in HSPM, with Ghanim et al reporting that about half of all children with HSPM had significant medical complications in the perinatal period (158). These included neonatal and delivery complications and low birth weight. The only perinatal event associated with HSPM according to Elfrink et al was low birth weight, which increased the odds of HSPM (157).

Early childhood illness

Several retrospective studies have reported that illness in the first three or four years of life is associated with MIH (153, 159). Although there is considerable variability in the definition of general health/illness, these studies seem to indicate some form of consensus.

Specifically, early childhood fever has been associated with higher odds of MIH in a number of retrospective studies that adjusted for confounding (154, 155). Ghanim et al also found a significant association in cases where fever was combined with other symptoms such as chest and/or ear infections. A prospective study of a cohort of German children found an association (aOR 2.48; 95% CI 1.35-4.56, $p<0.05$) between

respiratory disease and a more severe variant of MIH where incisor involvement was also present. Comparison between studies is difficult because there was variability in how asthma was defined, with some reporting on asthma in the first, first three and first four years of childhood and others combining asthma with other conditions such as allergy. In addition to asthma, pneumonia is another respiratory condition which has been found to increase the odds of MIH (154, 155). Other illnesses including measles, chicken pox, renal disease, gastrointestinal disease, bronchitis, tonsillitis, adenoiditis and otitis media have been implicated in the aetiology of MIH, but lack support from other studies (154, 155, 161-164).

Early childhood medication use

A recent systematic review found insufficient evidence to identify any drug as associated with MIH (165). However as MIH criteria were not applied to determine the eligibility of studies included in the review, the applicability of findings is questionable. Two retrospective studies found a strong association between antibiotic use in the first year of life and MIH (154, 166). Although the retrospective study by Allazam et al reported an association between antibiotic use anytime in early childhood and MIH (uOR 5.91, 95% CI 1.85-18.86), Pitiphat et al reported that the association did not remain significant when adjusted for confounding (uOR 2.5, 95% CI 1.2-5.2, aORs not provided) (159, 161).

In regards to specific antibiotics, both Laisi (prospective study, uOR 2.06, 95% CI 1.01-4.17) and Whatling and Fearne (retrospective study, uOR 5.22, 95% CI 1.11-5.89) reported a strong association with the use of amoxicillin (164, 167). However, Arrow did not find such an association when the data was stratified according to consumption at 0-1 years and 1-3 years (168). Souza et al reported a significant association when Amoxicillin was combined with other antibiotics, albeit only in rural locations (uOR 1.92, 95% CI 1.02-3.62) (153). The association with Amoxicillin alone was not assessed. Ghanim et al reported that the type of antibiotic did not demonstrate an association although there was no further information provided in this regard (154).

In a study using a Danish medication prescription database, Wogelius et al found that although anti-asthma medication was not associated with MIH (uOR 0.82, 95% CI 0.39-1.65), there may be an association with a subset of cases involving post-eruptive breakdown (uOR 2.42, 95% CI 0.70-7.43) (169). Using drug histories, Loli et al reported an association with aerosol therapy for respiratory diseases (uOR 3.19 95% CI 1.72 – 5.9) (170). In neither study was it possible to control for the illness itself, implying that asthma medication may actually be a proxy measure of disease. In the only other study to assess the relationship with asthma medication Arrow failed to find an association (uOR 1.17, 95% CI 0.51-2.26) (168).

Breastfeeding

In 1996 Finnish researchers suggested an association between breastfeeding and MIH as part of a study investigating the adverse effects of dioxins (150). The same group however, subsequently published studies showing that an association no longer existed, attributing this to reduction in the levels of dioxin pollution (171). All but one of the more recent studies, including both prospective and retrospective studies have also failed to any significant association. The only exception is a Swedish case-control study but as multiple regression was not conducted the association may be due to confounding by other factors (151).

Vitamin D

A cross-sectional study of a German cohort found that elevated levels of vitamin D at 10 years of age was associated with lower levels of MIH. However, as the formation of the FPM occurs much earlier in life, vitamin D at 10 years is unlikely to influence MIH lesions (172). A more recent study reporting a positive association between HSPM and bone mineral content adjusted for bone area at 6 years of age suggested that the systematic cause of HSPM may lead to deficiencies in mineralisation of bone (173). The lack of an association with MIH was explained by the prolonged duration of formation of the FPM compared to the second primary molar, which the authors suggest, would allow time for development of compensatory mechanisms. However, this hypothesis is inconsistent with the higher prevalence rates of MIH compared to HSPM.

Limitations of existing observational studies of MIH and HSPM

Overall, there are three major problems with the existing studies of MIH. The first is the lack of adjustment for confounding. As randomisation is impossible, the ability of confounders to exaggerate or diminish the importance of some factors is a recognised flaw of observational studies. This is generally overcome through various statistical methods, most commonly multiple regression, which are almost mandatory in such studies. However, a large number of studies make no attempt to adjust for potential confounders. Even where some form of adjustment was performed, this is reported poorly with confounders often not listed nor explained, and unadjusted and adjusted ORs and *P*-values not provided. Ideally, confounders should be based on existing evidence and the plausibility of an association with both the exposure of interest and the outcome, in this case, MIH.

The retrospective nature of most studies is another major problem with studies of MIH. As discussed by Alaluusua (2010), retrospective studies rely on parental recall, often many years after the event (147). There is strong evidence to indicate that mothers accurately recall perinatal factors such as gestational age, birth weight and mode of delivery, even many years after the events (174). However, some aspects of maternal health during pregnancy, recall of breastfeeding duration, child illness and medication use are less likely to be reliable (175, 176). A number of studies cited the difficulty in obtaining medical records and even these, unless kept in a standardised way, may lack the consistency and detail needed. Using a combination of the two has been shown to be most likely to yield accurate information. There are many barriers to conducting prospective studies, including cost, loss to follow-up, and non-participation. As such, using existing medical cohorts may provide the most practical means of conducting prospective studies. Nevertheless, the compatibility of such cohorts and specifically their inclusion and exclusion criteria with a study of MIH needs to be considered to ensure that all or at least most relevant exposures are included.

Finally, the lack of detail and consistency regarding the exposures investigated limits comparisons between studies. Further, it is likely that the study participants, often the mothers completing questionnaires regarding exposure to various environmental factors, were also given similarly basic details about the exposures and so the

accuracy of their responses are questionable. This may be partly due to the large number of exposures assessed in many studies, however in order to ensure the accuracy of the data collected, clear definitions of exposures investigated are recommended.

Possible mechanisms for an environmental aetiology

A number of biological mechanisms for the association between environmental factors and MIH/HSPM have been suggested, and are supported by laboratory and animal studies.

One theory regarding the cause of MIH is based on a concept of 'mineral toxicity' and is essentially, independent of an effect of ameloblasts. Intact MIH lesions have been found to be high in serum albumin (120). As albumin has been found in animal studies to halt mineralisation, it could potentially be the mechanism for the formation of MIH lesions (177). The cause or timing of albumin entry into the tooth is as yet unclear. There are three possible scenarios for timing; either pre-eruptively during tooth formation, during or after eruption. As the presence of enamel protein amelogenin prevents the attachment of albumin to the hydroxyapatite enamel crystal, it is thought to be protected during the secretory phase, which is high in these proteins. However, the period in late transition/early maturation, during which amelogenins are removed, may provide an opportunity for albumin entry into the tooth (119). Fever and inflammation lead to increased vascular permeability due to the release of various cytokines that act on endothelial cell membranes (178). Although lacking evidence, increased vascular permeability could account for the extravasation of proteins such as albumin into the developing FPMs.

Alternatively, MIH could result from altered ameloblast function, which is highly sensitive to changes in their surrounding environment, including changes induced by systemic illness (179). Recent *in vitro* rat studies show that prenatal exposure to endocrine disrupting chemicals (EDCs) can result in MIH-like lesions (180). The authors suggest that EDCs may increase expression of enamel proteins, reduce expression of the kallikrein 4 gene and lead to the accumulation of albumin, which hampers crystal growth. However, there are no observational studies investigating this link. Altered expression of genes important in enamel formation has also been

suggested as the link between fever and enamel defects. (181). Alternatively, the aetiology may relate to a metabolic disturbance such as described in rat studies showing that acidic conditions (a result of both localised inflammation and hypoxia) can affect crystal growth (182).

Part 3: The role of genes vs environment in dental caries

Increasingly the epidemiology of dental caries is polarising with specific sectors of the population found to be at highest risk. In Australia, the most affected 10% of 5 year olds have a mean DMFT (number of decayed. missing and filled teeth) of nearly ten, compared to a national average of nearly 2 (6). Along with other risk factors, genetic susceptibility might explain the why some small sections of community fail to benefit from preventive modalities that appear to be broadly effective. In addition, disease risk can vary between individuals despite similar environmental risk (183). Therefore, genetic influence in the pathogenesis of dental caries is likely. Similarly, as it is becoming increasingly apparent that no single environmental cause exists, there is a growing recognition that MIH and HSPM are likely to have complex aetiologies with a contribution from genetic factors (149).

Twin and Family Studies

Family studies can be used to determine genetic influences on a condition by assessing family aggregation. By comparing the rates of a condition amongst related individuals with rates in the general population, a relative risk ratio, a measure of the strength of aggregation, can be determined. However as the effect of the influence of shared environment may incorrectly present or be incorrectly interpreted as a genetic association. Nevertheless, use of sophisticated statistical methods have provided invaluable insight into the role of genetics in dental caries, and led the way for more recent DNA-based techniques.

Monozygotic (MZ) twins result from splitting of a single fertilised egg and in most cases are genetically identical. Dizygotic (DZ) twins arise when two separate eggs are fertilised by two separate spermatozoa and so, like other siblings in a family, share about half of their genetic composition. MZ and DZ twins are controlled for parents,

gestational age, age and some, for sex. And as most twin studies are based on twins reared together, share a range of other environmental exposures. The classic twin model compares the degree of variance of a condition within MZ pairs and DZ pairs to determine the importance of genetics (184). A purely genetic disease therefore has a correlation of 1 (or 100%) within MZ twins, but only 0.5 (or 50%) within DZ twins. If a disease is purely a result of the environment, the correlation of disease within MZ pairs will be similar to that within DZ pairs. The model is based on several assumptions, including that twins are representative of the general population and that the covariation in shared environments within MZ twins is the same as the covariation in shared environments for DZ twins. A further consideration within twin and family models is that for most diseases, neither genetics, nor environmental factors can explain all the variation in risk of a phenotype (or the presence or absence of disease) (185). This has been attributed to the role of non-shared environment. Therefore, the residual variation left after accounting for genetic susceptibility can be due to either shared (common) or non-shared (specific) factors that influence twins and indeed siblings differently (186). Statistical methods can be used to determine the relative contribution of each of these factors and this approach forms a platform for more detailed and specific analysis of the respective genetic, shared or non-shared environmental factors.

Twin and family studies of dental caries

Family and twin studies can be used to determine the heritability, that is, the genetic contribution to the variation in a phenotype, as a percentage from zero to 100 (187). A study of 2600 participants from 740 families found that although the development of carious lesions in both primary and permanent dentition caries had a genetic component, the effect was greater in the primary dentition, with a heritability of 54 – 70 % compared to the permanent dentition, with a heritability of 35 – 55% (188).

A series of recent studies from a high-caries risk Brazilian twin cohort with no access to water fluoridation and limited access to professional dental care has provided considerable evidence that dental caries is influenced by genetics (189, 190). Relatively complex statistical modelling methods were used to determine a heritability of 76.5 and 70.8 for surface based caries prevalence rates (SBCPR) and a

lesion severity index (LSI) respectively (189). A follow-up study investigating the incidence of dental caries in the cohort also reported a significant genetic component with a heritability of 30 for net change in SBCPR and 36.1 for net change in LSI (190). The authors do not comment on the significance of the markedly lower heritability estimates for caries incidence. An important note about heritability studies is that the reported values apply only to the conditions (e.g. environment, age, socioeconomic status) within the study. Therefore, the importance of genetics may be different in a cohort with access to water fluoridation. However, the authors argue that the true heritability of dental caries can only be studied when external factors such as fluoridation and professional dental caries are not part of the model. Additionally, as typified in the longitudinal study, where heritability for change in SBCPR ranged from 30 in the youngest age group (1.5 to 4-years) to 13.3 in 4 to 6 year old children and reached 46.3 in the children over six years of age, heritability can change depending on age. The change in heritability with time is a reflection of the change in the influence of environment. In the previous study, environmental factors appear to have more of an influence on the variance of the condition between 4 and 6 years of age, than the younger and older age groups. As such, focussing on heritability may be misguided when strong environment influences are present.

Although rare, studies of twins reared apart can provide valuable insight into the heritability of conditions. Calculating the variation in intra-class correlation for MZ and DZ twin pairs reared apart has enabled an estimate of heritability in a series of older studies (191, 192). A study of adult twins reared apart from childhood revealed a compellingly higher concordance for caries experience within MZ twins (ICC 0.79) compared to DZ twins (ICC 0.34) (191). A heritability index of 0.9 was suggestive of a strong genetic component in dental caries. A follow-up study that added to the data set showed this effect persisted, albeit in smaller magnitude with a heritability estimate of 0.64 (192).

Human Genetic studies

Advances in molecular genetics, especially with the advent of the human genome project, have enabled a more detailed assessment of the role of genetics in dental caries.

Genome-wide studies

Two genes located close to each other are likely to be linked, and therefore inherited together. Genetic linkage studies identify genetic loci by detecting genetic markers that are shared by family members with the condition but are not found in unaffected family members (193). The earliest genome wide scan was a linkage study conducted on 279 people from 64 families in Cambodia (194). Although the authors suggested that several chromosome locations may map to regions with genes important in saliva and immunity, no specific gene associations were reported, possibly because of the very low sample size. Linkage studies are useful in the analysis of highly penetrable diseases where one gene mutation leads to disease. However their use in complex and common diseases such as dental caries, where multiple genes are likely to be involved, is limited.

Genome-wide association studies (GWAS) survey the genomes of participants with different phenotypes (or with and without disease) to identify associated single nucleotide polymorphisms (SNPs). SNPs describe a single base change in the DNA sequence that is shared by more than 1% of the population, and represent gene loci of importance in disease risk (195). A GWAS of 518,997 genetic variants in 920 adults identified two significant genetic loci, *LYZL2* and *AJAP1*, important in host defence and tooth development respectively, as well as a number of suggestive loci (196). The same research group also conducted a GWAS of dental caries in 1305 children and despite failing find to significant loci, did report a number of suggestive loci (197). The authors suggested that the role of genetics in dental caries is complex and likely to be due to small contributions by a number of gene loci. A GWAS comparing caries in the pits and fissures and identified different suggestive gene loci, indicating that genetic influence is different between the two patterns of dental caries (198).

Candidate gene studies

Candidate gene studies statistically assess the association between genetic variation and disease for specific, hypotheses based genes. Candidate gene studies have reported some, albeit conflicting, evidence for an aetiological role for genes involved

in such caries risk factors as taste, immunity, saliva and tooth morphology and formation (183).

Problems with genetic studies of dental caries

Despite rapid developments in the field of genomics and bioinformatics, it is evident that there remains insufficient consistency in the description of the outcomes to identify specific genetic risk factors. This reflects a number of challenges, including the difficulty of establishing appropriate and consistent definitions for the phenotype (45). Vieira et al, in their genome-wide linkage study subdivided 'caries experience' into four different phenotypes spanning very low to high caries experience (194). Shaffer et al used five novel phenotypes in their GWAS, based on cluster analysis to identify patterns of surface predisposition to dental caries (55, 196). In their candidate gene study, Wendell et al dichotomised caries experience into those with carious lesions as opposed to those without (199). Slayton et al compared participants with DMFT of zero and those with a DMFT above three, excluding the intermediate group with DMFT of 1 - 3 (200). Despite the variations in phenotypes, all these studies are based on caries experience rather than active disease. The difficulty in interpreting whether a carious lesion is a site of active disease or simply a result of previous disease is an inherent issue in epidemiological, including genetic, studies of dental caries. Longitudinal studies that measure changes in caries increment are likely to be able to better identify individuals with active disease rather than studies utilising one off caries prevalence.

MIH/HSPM

As environmental factors have been the main focus of aetiological studies of MIH, genetic studies are lacking. There have been no family or twin studies of MIH or HSPM. A genome wide association study (GWAS) revealed a gene locus near SCUBE1 gene on chromosome 22 that may be important in MIH (206). However the study was underpowered and may have failed to identify important genome wide significant associations. A study of genes known to be involved in tooth development revealed an association between specific markers and MIH (207) . While some genetic variants increased the susceptibility to MIH, others appeared to be protective.

The authors suggest that an interplay between genetic and environmental factors explains the multifactorial aetiology of MIH/HSPM.

Epigenetics

The importance of epigenetics in complex diseases is increasingly recognised and may apply to dental caries and MIH/HSPM (201). Epigenetics describes the heritable modifications of genes, leading to differences in DNA expression within different tissues (83). Aside from cell lineage specification, epigenetics appear to mediate environmental influences on the gene expression. A study of several birth tissues in 56 MZ and 35 DZ newborn twins revealed that despite having identical DNA sequences and a degree of similarity in their epigenomes, MZ twins also have considerable discordance in epigenetic markers (202). Environmental mediation of epigenetic changes has been reported to occur from as early as the intra-uterine period, with a follow-up study of the same cohort indicating that non-shared intrauterine conditions, such as discordant placental weight and umbilical cord insertion could explain the discordance in epigenome (203). The most common epigenetic modification is gene methylation, addition of a methyl –CH group in CpG dinucleotides within the DNA sequence.

Despite recent interest, epigenetic dental studies are rare (204). However, a recent very small epigenome-wide association study identified differential methylation of nine genes involved in cartilage, bone, tooth and neural development that may explain hypodontia (205). Further studies investigating the role of epigenetic factors in dental caries would improve understanding of the aetiology of the condition and the mechanisms by which the disease is mediated by environmental factors.

Both a genetic predisposition and an environmental systemic cause would be expected to affect teeth in a stable, temporal pattern. However, in the case of MIH and HSPM, the cause must be able to explain why teeth forming at the same time can be affected to varying degrees. Therefore localised factors may influence the occurrence of MIH. Epigenetic factors can be site specific and could potentially impart different influences on teeth forming at the same time, albeit in different location. However, there are no epigenetic studies of MIH.

Conclusions

Oral diseases such as dental caries and MIH have a significant impact on Australian society due to their wide prevalence and impact on child health and development. Dental caries is a widely researched disease that continues to baffle with its persistence. MIH and HSPM are relatively recently described conditions with a rapidly growing evidence base that is nevertheless, incompletely understood, particularly in regards to aetiology and pathogenesis. In both conditions, early life is recognised to be a critical period in terms of disease risk. Understanding the importance of genetics and environment is a vital step in determining the aetiology of both dental caries and MIH/HSPM, where a combination of factors is likely to be important. This in turn, fosters the development of diagnostic and preventive measures that will effectively reduce the impact of these conditions. Reducing oral disease in childhood promises to not only improve oral health later in life but also contribute to improving overall health.

3 Methods & Conceptual Framework

3.1 PETS Cohort

The Peri/postnatal Epigenetic Twins Study (PETS) is a unique, longitudinal study of a birth cohort of 250 mothers and their twin children that was established in 2007. Women, pregnant with twins, were recruited mid-gestation and data collected on maternal nutrition and lifestyle risk at various time-points including the immediate post-natal period. The cohort was reviewed again when the twins were 18 months of age and information about health and nutrition and biological samples were collected. A summary of data collected as part of PETS is provided in figure 4.

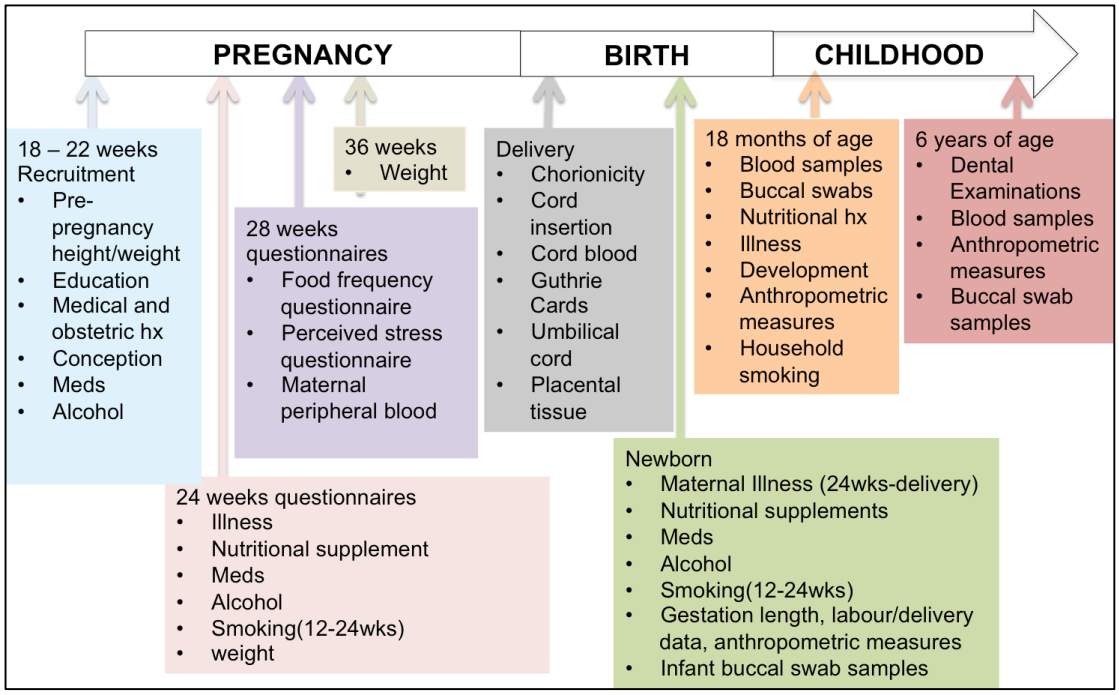


Figure 4: Summary of data collected in the PETS cohort

All 250 PETS twin pairs have been invited to participate in the current NIH-funded stage of the study, titled "What links dental health, heart health and gut health? A study of twin children" (#5R01DEO19655-02). Ethics approval was obtained from the Royal Children's Hospital Human Research Ethics Committee. Data is collected using online questionnaires and includes information regarding oral health, general health and diet. Families then attend the Royal Children's Hospital for a dental examination and collection of bio-samples.

3.2 Dental examination

Two trained and calibrated dental practitioners conduct a standardised comprehensive dental examination to assess soft and hard tissues, in particular dental caries and DDE including HSPM. Standard cross-infection protocol is adhered to, including the use of gloves and glasses. Dental caries is recorded using the International Caries Detection and Assessment System (ICDAS), which is a validated tool to record the depth of carious lesions. MIH/HSPM is measured using both the modified DDE Index (mDDE), a validated tool for epidemiological studies of DDE and a new MIH/HSPM specific criteria. While the mDDE is a broad index for all forms of DDE and has been used extensively in studies of DDE, the latter is a recently developed modification of the European Academy of Paediatric Dentistry Judgment Criteria, which allows for measurement of the nature, extent and severity of MIH/HSPM lesions.

3.3 Analysis Plan

Hypotheses 1 & 2

1. MZ and DZ twins are equally correlated for risk of dental caries.
2. MZ and DZ twins are equally correlated for risk of HSPM.

If the null hypothesis is rejected, variance components models will be fitted using the assumptions of the classic twin model to determine the contribution of additive polygenic (A) and shared (C) and non-shared (E) environmental factors to the variation in risk of dental caries and HSPM. If, suggestive of a lack of genetic influence, the null hypothesis cannot be rejected, the component due to additive polygenic effects (A) will be set to equal 0 and the contribution of shared (C) and non-shared environment (E) to the variation in risk of dental caries and HSPM will be estimated.

Hypotheses 3 & 4

3. Pre- and perinatal shared and non-shared factors are not associated with dental caries at age 6.
4. Pre- and perinatal shared and non-shared factors are not associated with HSPM.

A comprehensive list of both shared and unshared factors from pregnancy and early childhood that are potentially associated with risk of dental caries and MIH/HSPM will be investigated in this project (Table 1). The factors of interest have been identified through a comprehensive literature review of the aetiology of these conditions, including a systematic review of the aetiology of MIH/HSPM. Directed acyclic graphs are shown in Figure 5 and 6. Statistical analysis will involve within and between pair analysis as well as cross-sectional (non-twin specific) analysis of the cohort as individuals with appropriate adjustment for within pair correlation.

Table 1: Shared and non-shared factors

Shared factors
Maternal obesity
Maternal illness
Maternal stress
Maternal vitamin D
Maternal inflammation
Maternal smoking
Maternal alcohol intake
Medication during delivery
Delivery mode
Non-shared factors
Placental attachment
Intra-uterine growth restriction
Birth weight
Admission to NICU/SCN
Apgar Score
Duration of birth hospitalisation
Vitamin D at birth

Figure 5: Directed acyclic graph demonstrating proposed association between prenatal and perinatal factors and dental caries

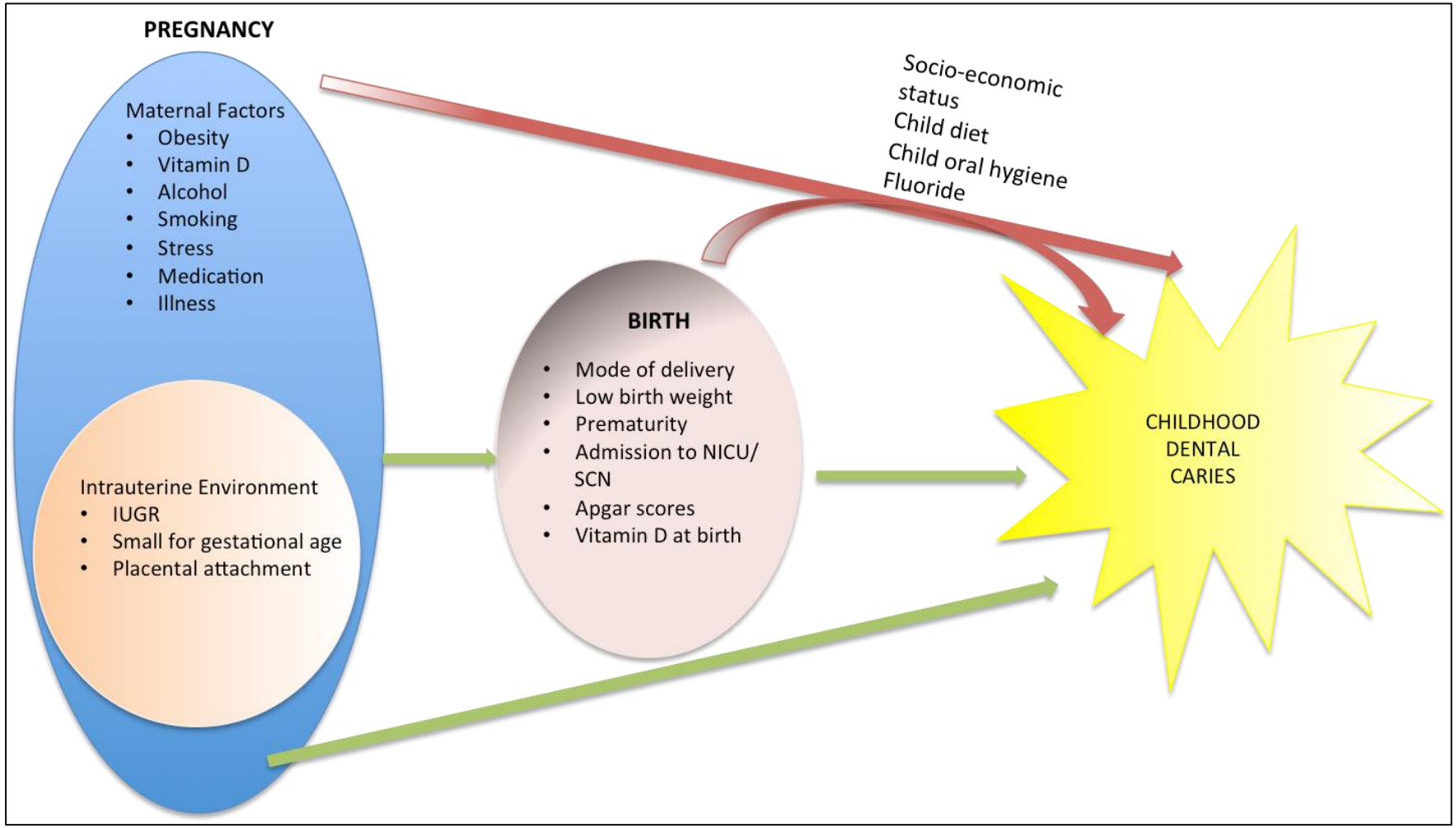
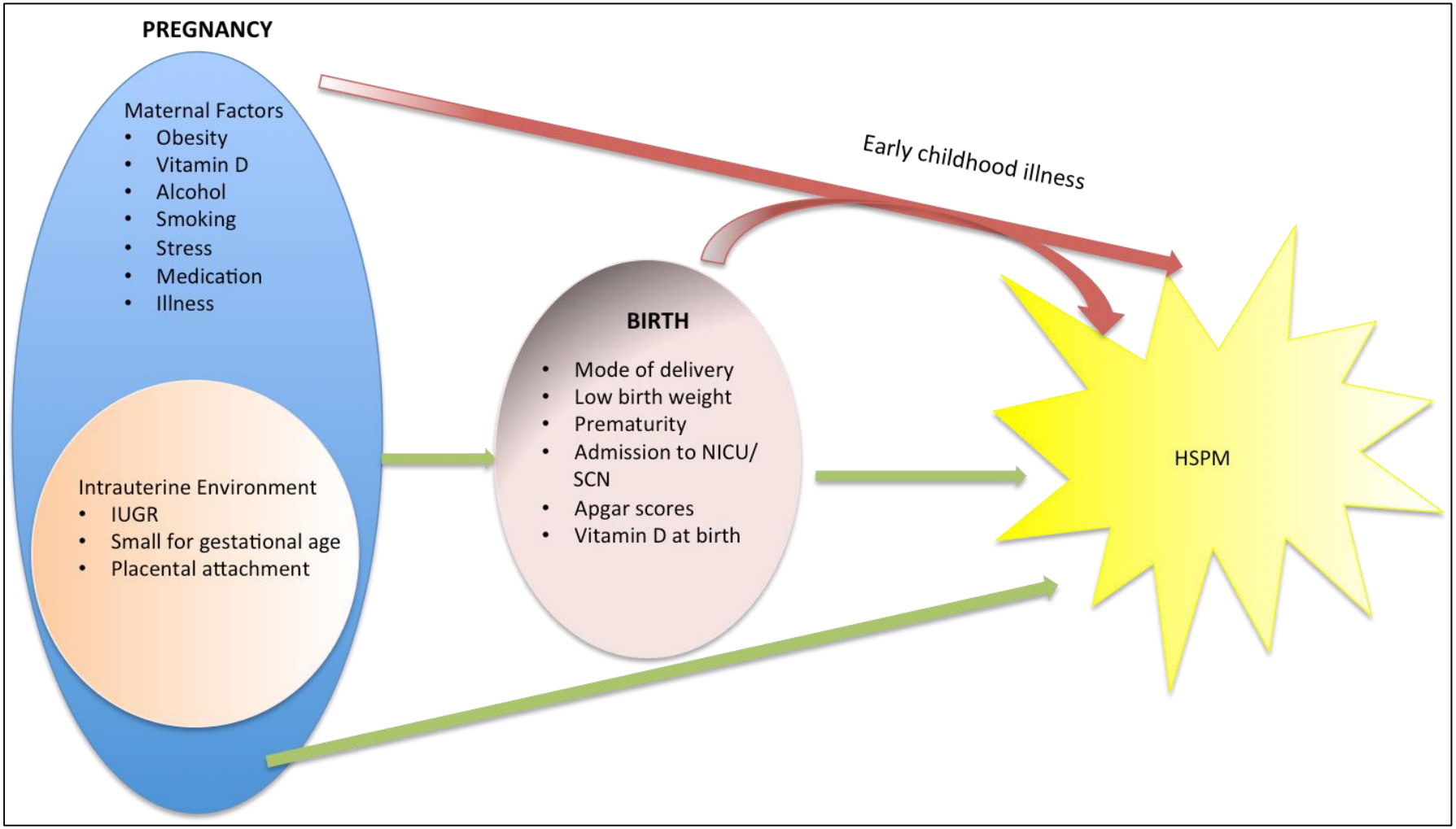


Figure 6: Directed acyclic graph demonstrating proposed association between prenatal and perinatal factors and dental HSPM



4. Preliminary data

4.1 Systematic Review

Aetiology of Molar Incisor Hypomineralisation – A Systematic Review

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Abstract

Objectives: Molar incisor hypomineralisation (MIH) is a common developmental dental defect of permanent teeth, which can increase the risk of dental caries, infection, and hospitalisation. The aetiology is currently unclear although prenatal or early childhood health factors are suspected. The aim of this systematic review was to assess the strength of evidence linking aetiological factors with MIH.

Methods: A systematic search was conducted using the Medline and Embase electronic databases for studies investigating environmental aetiological factors of MIH. Two reviewers assessed the eligibility of studies. The level of evidence and bias was determined for all eligible studies according to Australian National Health and Medical Research Council guidelines for systematic reviews of aetiology and the Newcastle-Ottawa Scale.

Results: From a total of 2254 studies identified through electronic and hand searching, 28 were eligible for inclusion. Twenty-five of these investigated MIH and three investigated a related condition in primary teeth, Hypomineralised second primary molars (HSPM) and these were analysed separately.

A limited number of studies reported significant associations between MIH and pre- and perinatal factors such as maternal illness and medication use in pregnancy, prematurity and birth complications. Early childhood illness was implicated as an aetiological factor in MIH in several studies, in particular fever, asthma and pneumonia. The studies investigating HSPM revealed an association with maternal

alcohol consumption, infantile fever and ethnicity. However, the validity of these findings is impaired by study design, lack of adjustment for confounders, lack of detail and consistency of exposures investigated and poor reporting.

Conclusions: Childhood illness is likely to be associated with MIH. Further prospective studies of the aetiology of MIH/HSPM are needed.

4.2 Data Checking and cleaning of exposure data

Extensive data checking and cleaning of the questionnaire data obtained from pregnancy and at 18 months of age has been completed.

5 Relevance and importance of the study

HSPM and dental caries are two common conditions affecting the health and well being of children, with lifelong implications. As dental caries and HSPM can present as significant clinical problems from a very young age, identifying early risk factors of the conditions is important. However, there is a scarcity of good quality evidence regarding the role of pre- and perinatal factors in the susceptibility to dental caries and HSPM. Prospective cohort studies are difficult and expensive to conduct, but provide the highest level of evidence for studies of aetiology where randomised controlled trials are not possible. With access to comprehensive, prospectively collected data from pregnancy (including biosamples), this study is uniquely placed to answer the important questions regarding the relationship between early life factors and oral disease in childhood.

As a twin cohort, this study will also be able to identify the contribution of genetic and shared and non-shared environment in HSPM and dental caries. This study is the first ever family study conducted on HSPM and will for the first time, clarify whether genetic factors are important in the condition.

Although non-shared environment has been recognised to be important in many complex behavioural diseases, its role in oral disease has not been widely researched. This study will be one of few to investigate the contribution of non-shared environment on the variation in risk of oral conditions.

Bibliography

1. Thomson W, Poulton R, Milne B, Caspi A, Broughton J, Ayers K. Socioeconomic inequalities in oral health in childhood and adulthood in a birth cohort. Community Dent Oral Epidemiol 2004;32:345-53.
2. Listl S, Galloway J, Mossey P, Marcenes W. Global Economic Impact of Dental Diseases. J Dent Res 2015:0022034515602879.
3. Friedewald VE, Kornman KS, Beck JD, Genco R, Goldfine A, Libby P, et al. The American Journal of Cardiology and Journal of Periodontology Editors' Consensus: Periodontitis and Atherosclerotic Cardiovascular Disease♦. J Periodontol 2009;80:1021-32.
4. World Health organisation. Global action plan for the prevention and control of noncommunicable diseases 2013-2020. Geneva, Switzerland: 2013.
5. Australian Institute of Health and Welfare. Dental decay among Australian children. Research report series no. 53. Canberra: Australian Instittue of health and welfare, 2011 Contract No.: Cat. no. DEN 210. .
6. Mejia G, Amarasena N, Ha D, Roberts-Thomson K, Ellershaw A. Child Dental Health Survey Australia 2007: 30-year trends in child oral health. Canberra: AIHW, 2012 Contract No.: Dental statistics and research series no.60. Cat. no. DEN 217.
7. Australian Institute of Health and Welfare. Health expenditure Australia 2008–09. Canberra
: AIHW, 2010 Contract No.: Health and welfare expenditure series no. 42. Cat. no. HWE 51.
8. Rogers J. Evidence-based oral health promotion resource. Melbourne: Prevention and Population Health Branch, Government of Victoria,
Department of Health, Prevention and Population Health Branch GoV, Health Do; 2011.
9. Slade GD, Spencer AJ, Davies MJ, Burrow D. Intra- oral distribution and impact of caries experience among South Australian school children. Aust Dent J 1996;41:343-50.
10. Finucane D. Rationale for restoration of carious primary teeth: a review. European archives of paediatric dentistry: official journal of the European Academy of Paediatric Dentistry 2012;13:281-92.
11. Poulton R, Caspi A, Milne BJ, Thomson WM, Taylor A, Sears MR, et al. Association between children's experience of socioeconomic disadvantage and adult health: a life-course study. The Lancet 2002;360:1640-5.
12. Ruby J, Goldner M. Nature of symbiosis in oral disease. J Dent Res 2007;86:8-11.
13. Featherstone J. Dental caries: a dynamic disease process. Aust Dent J 2008;53:286-91.
14. Manton D. Dental caries: where to from here? Ann R Australas Coll Dent Surg 2008;19:73.
15. Featherstone J. The continuum of dental caries—evidence for a dynamic disease process. J Dent Res 2004;83:C39-C42.
16. Bjørndal L, Darvann T, Thylstrup A. A quantitative light microscopic study of the odontoblast and subodontoblastic reactions to active and arrested enamel caries without cavitation. Caries Res 1997;32:59-69.

17. Selwitz RH, Ismail AI, Pitts NB. Dental caries. The Lancet 2007;369:51-9.
18. Fejerskov O. Changing paradigms in concepts on dental caries: consequences for oral health care. Caries Res 2004;38:182-91.
19. Cephas KD, Kim J, Mathai RA, Barry KA, Dowd SE, Meline BS, et al. Comparative analysis of salivary bacterial microbiome diversity in edentulous infants and their mothers or primary care givers using pyrosequencing. PLoS One 2011;6:e23503-e.
20. Lazarevic V, Whiteson K, Hernandez D, François P, Schrenzel J. Study of inter-and intra-individual variations in the salivary microbiota. BMC Genomics 2010;11:523.
21. Luo A, Yang D, Xin B, Paster B, Qin J. Microbial profiles in saliva from children with and without caries in mixed dentition. Oral Dis 2012;18:595-601.
22. Nasidze I, Li J, Quinque D, Tang K, Stoneking M. Global diversity in the human salivary microbiome. Genome Res 2009;19:636-43.
23. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome Biol 2012;13:R42.
24. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res 2012;22:2146-52.
25. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. bioRxiv 2016:036103.
26. Zaura E, ten Cate JM. Towards Understanding Oral Health. Caries Res 2015;49:55-61.
27. Marsh PD, Head DA, Devine DA. Ecological Approaches to Oral Biofilms: Control without Killing. Caries Res 2015;49:46-54.
28. Wade WG. The oral microbiome in health and disease. Pharmacol Res 2013;69:137-43.
29. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy. BMC Microbiol 2009;9:259.
30. Rautava S, Collado MC, Salminen S, Isolauri E. Probiotics modulate host-microbe interaction in the placenta and fetal gut: a randomized, double-blind, placebo-controlled trial. Neonatology 2012;102:178-84.
31. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy, intestinal colonization and human disease. Nature Reviews Gastroenterology and Hepatology 2012;9:565-76.
32. Zhu M, Du M, Nathanielsz P, Ford S. Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. Placenta 2010;31:387-91.
33. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences 2010;107:11971-5.
34. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell 2014;159:789-99.
35. Balakrishnan M, Simmonds RS, Tagg JR. Dental caries is a preventable infectious disease. Aust Dent J 2000;45:235-45.
36. Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. Caries Res 2008;42:409-18.

37. Takahashi N, Nyvad B. The role of bacteria in the caries process ecological perspectives. J Dent Res 2011;90:294-303.
38. Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA, et al. Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol 2010;48:4121-8.
39. Johansson I, Witkowska E, Kaveh B, Holgerson PL, Tanner A. The Microbiome in Populations with a Low and High Prevalence of Caries. J Dent Res 2016;95:80-6.
40. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008;46:1407-17.
41. Beighton D. The complex oral microflora of high- risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005;33:248-55.
42. Kleinberg I. A Mixed-Bacteria Ecological Approach to Understanding the Role of the Oral Bacteria in Dental Caries Causation: An Alternative to Streptococcus Mutans and the Specific-Plaque Hypothesis. Crit Rev Oral Biol Med 2002;13:108-25.
43. Simón-Soro A, Tomás I, Cabrera-Rubio R, Catalan M, Nyvad B, Mira A. Microbial geography of the oral cavity. J Dent Res 2013;92:616-21.
44. Thomas R, Zijnge V, Ciçek A, De Soet J, Harmsen H, Huysmans M. Shifts in the Microbial Population in Relation to in situ Caries Progression. Caries Res 2012;46:427-31.
45. Vieira AR, Modesto A, Marazita ML. Caries: Review of Human Genetics Research. Caries Res 2014;48:491-506.
46. Lehner T, Lamb JR, Welsh KL, Batchelor RJ. Association between HLA-DR antigens and helper cell activity in the control of dental caries. 1981.
47. Julihn A, Ekbom A, Modéer T. Maternal overweight and smoking: prenatal risk factors for caries development in offspring during the teenage period. Eur J Epidemiol 2009;24:753-62.
48. Schroth RJ, Lavelle C, Tate R, Bruce S, Billings RJ, Moffatt ME. Prenatal Vitamin D and Dental Caries in Infants. Pediatrics 2014;133:e1277-e84.
49. Wigen TI, Wang NJ. Maternal health and lifestyle, and caries experience in preschool children. A longitudinal study from pregnancy to age 5 yr. Eur J Oral Sci 2011;119:463-8.
50. Leme AP, Koo H, Bellato C, Bedi G, Cury J. The role of sucrose in cariogenic dental biofilm formation—new insight. J Dent Res 2006;85:878-87.
51. Takahashi N, Washio J. Metabolomic effects of xylitol and fluoride on plaque biofilm in vivo. J Dent Res 2011;90:1463-8.
52. Burne R, Zeng L, Ahn S, Palmer S, Liu Y, Lefebure T, et al. Progress dissecting the oral microbiome in caries and health. Adv Dent Res 2012;24:77-80.
53. De Stoppelaar J, Van Houte J, Backer Dirks O. The effect of carbohydrate restriction on the presence of Streptococcus mutans, Streptococcus sanguis and iodophilic polysaccharide-producing bacteria in human dental plaque. Caries Res 1970;4:114-23.
54. Palmer CA, Kent R, Jr., Loo CY, Hughes CV, Stutius E, Pradhan N, et al. Diet and caries-associated bacteria in severe early childhood caries. J Dent Res 2010;89:1224-9.
55. Shaffer J, Feingold E, Wang X, Weeks D, Weyant R, Crout R, et al. Clustering tooth surfaces into biologically informative caries outcomes. J Dent Res 2012:0022034512463241.

56. Batchelor PA, Sheiham A. Grouping of tooth surfaces by susceptibility to caries: a study in 5–16 year-old children. BMC Oral Health 2004;4:2.
57. Psoter WJ, Zhang H, Pendrys DG, Morse DE, Mayne ST. Classification of dental caries patterns in the primary dentition: a multidimensional scaling analysis. Community Dent Oral Epidemiol 2003;31:231-8.
58. Li Y, Navia JM, Bian JY. Caries experience in deciduous dentition of rural Chinese children 3-5 years old in relation to the presence or absence of enamel hypoplasia. Caries Res 1996;30:8-15.
59. Moynihan P, Kelly S. Effect on caries of restricting sugars intake systematic review to inform WHO guidelines. J Dent Res 2013:0022034513508954.
60. Guideline: sugars intake for adults and children. Geneva: World Health Organisation, 2015.
61. Sheiham A, James W. Diet and Dental Caries The Pivotal Role of Free Sugars Reemphasized. J Dent Res 2015;94:1341-7.
62. Burt BA, Pai S. Sugar consumption and caries risk: a systematic review. J Dent Educ 2001;65:1017-23.
63. Gustafsson BE, Quensel C-E, Lanke LS, Lundqvist C, Grahnen H, Bonow BE, et al. The effect of different levels of carbohydrate intake on caries activity in 436 individuals observed for five years. Acta Odontol Scand 1953;11:232-364.
64. Scheinin A, Mäkinen KK. Turku sugar studies: An overview. Acta Odontol Scand 1976;34:405-8.
65. Jamel H, Plasschaert A, Sheiham A. Dental caries experience and availability of sugars in Iraqi children before and after the United Nations sanctions. Int Dent J 2004;54:21-5.
66. Takeuchi M. Epidemiological study on dental caries in Japanese children before, during and after World War II. Int Dent J 1961;11:443-57.
67. Bowen W, Eastoe J, Cock D. The effect of sugar solutions on the pH of plaque in caries-active monkeys (Macaca irus). Arch Oral Biol 1966;11:833-IN15.
68. Stephan RM. Intra-oral hydrogen-ion concentrations associated with dental caries activity. J Dent Res 1944;23:257-66.
69. Aires C, Tabchoury C, Del Bel Cury A, Koo H, Cury J. Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. Caries Res 2006;40:28-32.
70. Leme AP, Dalcico R, Tabchoury C, Cury ADB, Rosalen P, Cury J. In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after APF application and F dentifrice use. J Dent Res 2004;83:71-5.
71. Lingstrom P, Van Houte J, Kashket yS. Food starches and dental caries. Crit Rev Oral Biol Med 2000;11:366-80.
72. Campain AC, Morgan MV, Evans RW, Ugoni A, Adams GG, Conn JA, et al. Sugar–starch combinations in food and the relationship to dental caries in low- risk adolescents. Eur J Oral Sci 2003;111:316-25.
73. Kashket S, Zhang J, Van Houte J. Accumulation of fermentable sugars and metabolic acids in food particles that become entrapped on the dentition. J Dent Res 1996;75:1885-91.
74. Linke HB, Birkenfeld L. Clearance and metabolism of starch foods in the oral cavity. Ann Nutr Metab 1999;43:131-9.
75. Chankanka O, Marshall TA, Levy SM, Warren JJ, Broffitt MB, Kolker JL, et al. Mixed dentition cavitated caries incidence and dietary intake frequencies. Pediatr Dent 2011;33:233.

76. Bradshaw DJ, Lynch RJ. Diet and the microbial aetiology of dental caries: new paradigms. Int Dent J 2013;63:64-72.
77. Reynolds E, Johnson I. Effect of milk on caries incidence and bacterial composition of dental plaque in the rat. Arch Oral Biol 1981;26:445-51.
78. Dashper SG, Saion BN, Stacey MA, Manton DJ, Cochrane NJ, Stanton DP, et al. Acidogenic potential of soy and bovine milk beverages. J Dent 2012;40:736-41.
79. Anderson C, Curzon M, Van Loveren C, Tatsi C, Duggal M. Sucrose and dental caries: a review of the evidence. Obes Rev 2009;10:41-54.
80. Harris R, Nicoll AD, Adair PM, Pine CM. Risk factors for dental caries in young children: a systematic review of the literature. Community Dent Health 2004;21:71-85.
81. Slade G, Sanders A, Do L, Roberts-Thomson K, Spencer A. Effects of Fluoridated Drinking Water on Dental Caries in Australian Adults. J Dent Res 2013.
82. Leong PM, Gussy MG, Barrow SYL, Silva- Sanigorski A, Waters E. A systematic review of risk factors during first year of life for early childhood caries. Int J Paediatr Dent 2013;23:235-50.
83. Barros S, Offenbacher S. Epigenetics: connecting environment and genotype to phenotype and disease. J Dent Res 2009;88:400-8.
84. Gluckman PD, Hanson MA, Buklijas T. A conceptual framework for the developmental origins of health and disease. J Dev Orig Health Dis 2010;1:6-18.
85. Seow W. Developmental defects of enamel and dentine: challenges for basic science research and clinical management. Aust Dent J 2014;59:143-54.
86. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266-81.
87. Hujoel PP. Vitamin D and dental caries in controlled clinical trials: systematic review and meta-analysis. Nutr Rev 2013;71:88-97.
88. Schroth RJ, Levi JA, Sellers EA, Friel J, Kliewer E, Moffatt ME. Vitamin D status of children with severe early childhood caries: a case–control study. BMC Pediatr 2013;13:1.
89. Tanaka K, Hitsumoto S, Miyake Y, Okubo H, Sasaki S, Miyatake N, et al. Higher vitamin D intake during pregnancy is associated with reduced risk of dental caries in young Japanese children. Ann Epidemiol 2015;25:620-5.
90. Herzog K, Scott JM, Hujoel P, Seminario AL. Association of vitamin D and dental caries in children: Findings from the National Health and Nutrition Examination Survey, 2005-2006. The Journal of the American Dental Association 2016.
91. Griffin MD, Xing N, Kumar R. Vitamin D and its analogs as regulators of immune activation and antigen presentation. Annu Rev Nutr 2003;23:117-45.
92. Aligne CA, Moss ME, Auinger P, Weitzman M. Association of pediatric dental caries with passive smoking. JAMA 2003;289:1258-64.
93. Williams S, Kwan S, Parsons S. Parental Smoking Practices and Caries Experience in Pre–School Children. Caries Res 2000;34:117-22.
94. Hanioka T, Ojima M, Tanaka K, Yamamoto M. Does secondhand smoke affect the development of dental caries in children? A systematic review. Int J Environ Res Public Health 2011;8:1503-19.
95. Iida H, Auinger P, Billings RJ, Weitzman M. Association between infant breastfeeding and early childhood caries in the United States. Pediatrics 2007;120:e944-e52.
96. Tanaka S, Shinzawa M, Tokumasu H, Seto K, Tanaka S, Kawakami K. Secondhand smoke and incidence of dental caries in deciduous teeth among children in Japan: population based retrospective cohort study. BMJ 2015;351:h5397.

97. Dong Q, Wu H, Dong G, Lou B, Yang L, Zhang L. The morphology and mineralization of dental hard tissue in the offspring of passive smoking rats. Arch Oral Biol 2011;56:1005-13.
98. Wu Q, Suzuki M. Parental obesity and overweight affect the body- fat accumulation in the offspring: the possible effect of a high- fat diet through epigenetic inheritance. Obes Rev 2006;7:201-8.
99. Hayden C, Bowler JO, Chambers S, Freeman R, Humphris G, Richards D, et al. Obesity and dental caries in children: a systematic review and meta- analysis. Community Dent Oral Epidemiol 2013;41:289-308.
100. Sayyed T, Kandil M, Bashir O, Alnaser H. The relationship between term pre-eclampsia and the risk of early childhood caries. The Journal of Maternal-Fetal & Neonatal Medicine 2014;27:62-5.
101. Ramos-Gomez F, Weintraub J, Gansky S, Hoover C, Featherstone J. Bacterial, behavioral and environmental factors associated with early childhood caries. J Clin Pediatr Dent 2003;26:165-73.
102. World Health Organisation. International statistical classification of diseases and related health problems. 2010.
103. Australian Institute of Health and Welfare. Determinants key indicators - Low birth weight [cited 2016 5 Feb 2016]. Available from: http://www.aihw.gov.au/chronic-diseases/key-indicators/determinants/.
104. Nelson S, Albert JM, Lombardi G, Wishnek S, Asaad G, Kirchner HL, et al. Dental caries and enamel defects in very low birth weight adolescents. Caries Res 2010;44:509-18.
105. Shulman J. Is there an association between low birth weight and caries in the primary dentition? Caries Res 2005;39:161-7.
106. Burt BA, Pai S. Does low birthweight increase the risk of caries? A systematic review. J Dent Educ 2001;65:1024-7.
107. Massoni AC, Chaves AM, Rosenblatt A, Sampaio FC, Oliveira AF. Prevalence of enamel defects related to pre-, peri- and postnatal factors in a Brazilian population. Community Dent Health 2009;26:143-9.
108. Kramer MS. Determinants of low birth weight: methodological assessment and meta-analysis. Bull World Health Organ 1987;65:663.
109. Saraiva MC, Bettiol H, Barbieri MA, Silva AA. Are intrauterine growth restriction and preterm birth associated with dental caries? Community Dent Oral Epidemiol 2007.
110. Alaki SM, Burt BA, Garetz SL. The association between antibiotics usage in early childhood and early childhood caries. Pediatr Dent 2009;31:31-7.
111. Nelun Barfod M, Magnusson K, Lexner MO, Blomqvist S, Dahlén G, Twetman S. Oral microflora in infants delivered vaginally and by caesarean section. Int J Paediatr Dent 2011;21:401-6.
112. Li Y, Caufield P, Dasanayake A, Wiener H, Vermund S. Mode of delivery and other maternal factors influence the acquisition of Streptococcus mutans in infants. J Dent Res 2005;84:806-11.
113. Barfod MN, Christensen LB, Twetman S, Lexner MO. Caries prevalence in Danish pre-school children delivered vaginally and by caesarean section. Acta Odontol Scand 2012;70:190-3.
114. Pattanaporn K, Saraithong P, Khongkhunthian S, Aleksejuniene J, Laohapensang P, Chhun N, et al. Mode of delivery, mutans streptococci colonization, and early childhood caries in three- to five- year- old Thai children. Community Dent Oral Epidemiol 2013;41:212-23.

115. Weerheijm KL, Jalevik B, Alaluusua S. Molar-incisor hypomineralisation. Caries Res 2001;35:390-1.
116. Ghanim A, Manton D, Marino R, Morgan M, Bailey D. Prevalence of demarcated hypomineralisation defects in second primary molars in Iraqi children. Int J Paediatr Dent 2013;23:48-55.
117. Elfrink MEC, ten Cate JM, Jaddoe VWV, Hofman A, Moll HA, Veerkamp JSJ. Deciduous Molar Hypomineralization and Molar Incisor Hypomineralization. J Dent Res 2012;91:551-5.
118. Crombie FA, Manton DJ, Palamara JE, Zalizniak I, Cochrane NJ, Reynolds EC. Characterisation of developmentally hypomineralised human enamel. J Dent 2013;41:611-8.
119. Farah RA, Monk BC, Swain MV, Drummond BK. Protein content of molar–incisor hypomineralisation enamel. J Dent 2010;38:591-6.
120. Mangum JE, Crombie FA, Kilpatrick N, Manton DJ, Hubbard MJ. Surface integrity governs the proteome of hypomineralized enamel. J Dent Res 2010;89:1160-5.
121. Mahoney EK, Rohanizadeh R, Ismail F, Kilpatrick N, Swain M. Mechanical properties and microstructure of hypomineralised enamel of permanent teeth. Biomaterials 2004;25:5091-100.
122. Jalevik B, Noren JG. Enamel hypomineralization of permanent first molars: a morphological study and survey of possible aetiological factors. Int J Paediatr Dent 2000;10:278-89.
123. Fagrell T. Molar incisor hypomineralization. Morphological and chemical aspects, onset and possible etiological factors. Swedish Dental Journal - Supplement 2011:5, 11-83.
124. Costa- Silva D, Cristiane M, Ambrosano G, Jeremias F, Souza D, Juliana F, et al. Increase in severity of molar–incisor hypomineralization and its relationship with the colour of enamel opacity: a prospective cohort study. Int J Paediatr Dent 2011;21:333-41.
125. da Costa-Silva CM, Jeremias F, de Souza JF, Cordeiro RdCL, Santos-Pinto L, Zuanon ACC. Molar incisor hypomineralization: prevalence, severity and clinical consequences in Brazilian children. Int J Paediatr Dent 2010;20:426-34.
126. Ghanim A, Morgan M, Marino R, Bailey D, Manton D. Molar-incisor hypomineralisation: prevalence and defect characteristics in Iraqi children. Int J Paediatr Dent 2011;21:413-21.
127. Weerheijm KL. Molar incisor hypomineralization (MIH): clinical presentation, aetiology and management. Dent Update 2004;31:9-12.
128. Weerheijm KL. Molar incisor hypomineralisation (MIH). Eur J Paediatr Dent 2003;4:114-20.
129. Kuhnisch J, Heitmuller D, Thiering E, Brockow I, Hoffmann U, Neumann C, et al. Proportion and extent of manifestation of molar-incisor-hypomineralizations according to different phenotypes. J Public Health Dent 2014;74:42-9.
130. Leen A. The interrelationship between molar hypomineralisation and orthodontics. [Thesis]. Melbourne: The University of Melbourne.; 2013.
131. Mittal N, Sharma B. Hypomineralised second primary molars: prevalence, defect characteristics and possible association with Molar Incisor Hypomineralisation in Indian children. Eur Arch Paediatr Dent 2015:1-7.
132. Ghanim A, Elfrink ME, Weerheijm K, Marino R, Manton DJ. A Practical Method for Use in Epidemiologic Studies on enamel hypomineralisation. Eur Arch Paediatr Dent 2015;16:235-46.

133. Weerheijm KL, Duggal M, Mejare I, Papagiannoulis L, Koch G, Martens LC, et al. Judgement criteria for molar incisor hypomineralisation (MIH) in epidemiologic studies: a summary of the European meeting on MIH held in Athens, 2003. Eur J Paediatr Dent 2003;4:110-3.
134. Arrow P. Prevalence of developmental enamel defects of the first permanent molars among school children in Western Australia. Aust Dent J 2008;53:250-9.
135. Jalevik B. Prevalence and Diagnosis of Molar-Incisor- Hypomineralisation (MIH): A systematic review. Eur Arch Paediatr Dent 2010;11:59-64.
136. Elfrink MEC, Schuller AA, Weerheijm KL, Veerkamp JSJ. Hypomineralized second primary molars: prevalence data in Dutch 5-year-olds. Caries Res 2008;42:282-5.
137. Owen M. Prevalence of early childhood caries and enamel defects in three to five year old Melbourne preschool children [Thesis]. Melbourne: The University of Melbourne; 2015.
138. Crombie FA, Manton DJ, Weerheijm KL, Kilpatrick NM. Molar incisor hypomineralization: a survey of members of the Australian and New Zealand Society of Paediatric Dentistry. Aust Dent J 2008;53:160-6.
139. Jalevik B, Klingberg GA. Dental treatment, dental fear and behaviour management problems in children with severe enamel hypomineralization of their permanent first molars. Int J Paediatr Dent 2002;12:24-32.
140. Lygidakis NA, Wong F, Jalevik B, Vierrou AM, Alaluusua S, Espelid I. Best Clinical Practice Guidance for clinicians dealing with children presenting with Molar-Incisor-Hypomineralisation (MIH): An EAPD Policy Document. Eur Arch Paediatr Dent 2010;11:75-81.
141. Jalevik B, Klingberg G. Treatment outcomes and dental anxiety in 18-year-olds with MIH, comparisons with healthy controls - a longitudinal study. Int J Paediatr Dent 2012;22:85-91.
142. Rodd H, Abdul- Karim A, Yesudian G, O'Mahony J, Marshman Z. Seeking children's perspectives in the management of visible enamel defects. Int J Paediatr Dent 2011;21:89-95.
143. Ong DV, Bleakley J. Compromised first permanent molars: an orthodontic perspective. Aust Dent J 2010;55:2-14.
144. Elfrink MEC, Schuller AA, Veerkamp JSJ, Poorterman JHG, Moll HA, Ten Cate BM. Factors increasing the caries risk of second primary molars in 5-year-old Dutch children. Int J Paediatr Dent 2010;20:151-7.
145. Leppaniemi A, Lukinmaa PL, Alaluusua S. Nonfluoride hypomineralizations in the permanent first molars and their impact on the treatment need. Caries Res 2001;35:36-40.
146. Heitmuller D, Thiering E, Hoffmann U, Heinrich J, Manton D, KÜHNISCH J, et al. Is there a positive relationship between molar incisor hypomineralisations and the presence of dental caries? Int J Paediatr Dent 2012.
147. Alaluusua S. Aetiology of molar-incisor hypomineralisation: a systematic review. Eur Arch Paediatr Dent 2010;11:53-8.
148. Crombie F, Manton D, Kilpatrick N. Aetiology of molar-incisor hypomineralization: a critical review. Int J Paediatr Dent 2009;19:73-83.
149. Lygidakis NA, Dimou G, Marinou D. Molar-incisor-hypomineralisation (MIH). A retrospective clinical study in Greek children. II. Possible medical aetiological factors. Eur Arch Paediatr Dent 2008;9:207-17.

150. Alaluusua S, Lukinmaa PL, Koskimies M, Pirinen S, Hölttä P, Kallio M, et al. Developmental dental defects associated with long breast feeding. Eur J Oral Sci 1996;104:493-7.
151. Fagrell TG, Ludvigsson J, Ullbro C, Lundin S-A, Koch G. Aetiology of severe demarcated enamel opacities--an evaluation based on prospective medical and social data from 17,000 children. Swed Dent J 2010;35:57-67.
152. Kuhnisch J, Mach D, Thiering E, Brockow I, Hoffmann U, Neumann C, et al. Respiratory diseases are associated with molar-incisor hypomineralizations. Swiss Dent J 2014;124:286-93.
153. Souza JF, Costa-Silva CM, Jeremias F, Santos-Pinto L, Zuanon AC, Cordeiro RC. Molar incisor hypomineralisation: possible aetiological factors in children from urban and rural areas. Eur Arch Paediatr Dent 2012;13:164-70.
154. Ghanim A, Manton D, Bailey D, Marino R, Morgan M. Risk factors in the occurrence of molar–incisor hypomineralization amongst a group of Iraqi children. Int J Paediatr Dent 2013;23:197-206.
155. Sönmez H, Yıldırım G, Bezgin T. Putative factors associated with molar incisor hypomineralisation: an epidemiological study. Eur Arch Paediatr Dent 2013;14:375-80.
156. Elfrink ME, Moll HA, Kiefte-de Jong JC, El Marroun H, Jaddoe VW, Hofman A, et al. Is Maternal Use of Medicines during Pregnancy Associated with Deciduous Molar Hypomineralisation in the Offspring? A Prospective, Population-Based Study. Drug Saf 2013;36:627-33.
157. Elfrink ME, Moll HA, Kiefte-de Jong JC, Jaddoe VW, Hofman A, Jacob M, et al. Pre-and Postnatal Determinants of Deciduous Molar Hypomineralisation in 6-Year-Old Children. The Generation R Study. PLoS One 2014;9:e91057.
158. Ghanim AM, Morgan MV, Marino RJ, Bailey DL, Manton DJ. Risk factors of hypomineralised second primary molars in a group of Iraqi schoolchildren. Eur Arch Paediatr Dent 2012;13:111-8.
159. Pitiphat W, Luangchaichaweng S, Pungchanchaikul P, Angwaravong O, Chansamak N. Factors associated with molar incisor hypomineralization in Thai children. Eur J Oral Sci 2014;122:265-70.
160. Brogårdh- Roth S, Matsson L, Klingberg G. Molar- incisor hypomineralization and oral hygiene in 10- to- 12- yr- old Swedish children born preterm. Eur J Oral Sci 2011;119:33-9.
161. Allazzam SM, Alaki SM, El Meligy OAS. Molar Incisor Hypomineralization, Prevalence, and Etiology. International journal of dentistry 2014;2014.
162. Beentjes V, Weerheijm K, Groen H. Factors involved in the aetiology of molar-incisor hypomineralisation (MIH). Eur J Paediatr Dent 2002;3:9-13.
163. Kusku OO, Caglar E, Sandalli N. The prevalence and aetiology of molar-incisor hypomineralisation in a group of children in Istanbul. Eur J Paediatr Dent 2008;9:139-44.
164. Whatling R, Fearne JM. Molar incisor hypomineralization: a study of aetiological factors in a group of UK children*. Int J Paediatr Dent 2008;18:155-62.
165. Serna C, Vicente A, Finke C, Ortiz AJ. Drugs related to the etiology of molar incisor hypomineralization: A systematic review. The Journal of the American Dental Association 2015.
166. Jalevik B, Noren JG, Klingberg G, Barregard L. Etiologic factors influencing the prevalence of demarcated opacities in permanent first molars in a group of Swedish children. Eur J Oral Sci 2001;109:230-4.

167. Laisi S, Ess A, Sahlberg C, Arvio P, Lukinmaa PL, Alaluusua S. Amoxicillin may cause molar incisor hypomineralization. J Dent Res 2009;88:132-6.
168. Arrow P. Risk factors in the occurrence of enamel defects of the first permanent molars among schoolchildren in Western Australia. Community Dent Oral Epidemiol 2009;37:405-15.
169. Wogelius P, Haubek D, Nechifor A, Nørgaard M, Tvedebrink T, Poulsen S. Association between use of asthma drugs and prevalence of demarcated opacities in permanent first molars in 6- to- 8- year- old Danish children. Community Dent Oral Epidemiol 2010;38:145-51.
170. Loli D, Costacurta M, Maturo P, Docimo R. Correlation between aerosol therapy in early childhood and Molar Incisor Hypomineralisation. Eur J Paediatr Dent 2015;16:73-7.
171. Laisi S, Kiviranta H, Lukinmaa PL, Vartiainen T, Alaluusua S. Molar-incisor-hypomineralisation and dioxins: new findings. Eur Arch Paediatr Dent 2008;9:224-7.
172. Kühnisch J, Thiering E, Kratzsch J, Heinrich-Weltzien R, Hickel R, Heinrich J, et al. Elevated Serum 25 (OH)-Vitamin D Levels Are Negatively Correlated with Molar-Incisor Hypomineralization. J Dent Res 2015;94:381-7.
173. van der Tas J, Elfrink M, Vucic S, Heppe D, Veerkamp J, Jaddoe V, et al. Association between Bone Mass and Dental Hypomineralization. J Dent Res 2016:0022034515625470.
174. Rice F, Lewis A, Harold G, van den Bree M, Boivin J, Hay DF, et al. Agreement between maternal report and antenatal records for a range of pre and peri-natal factors: the influence of maternal and child characteristics. Early Hum Dev 2007;83:497-504.
175. Liu J, Tuvblad C, Li L, Raine A, Baker LA. Medical record validation of maternal recall of pregnancy and birth events from a twin cohort. Twin Research and Human Genetics 2013;16:845-60.
176. Tienboon P, Rutishauser IH, Wahlqvist ML. Maternal recall of infant feeding practices after an interval of 14 to 15 years. Australian Journal of Nutrition and Dietetics 1994;51:25-.
177. Robinson C, Kirkham J, Brookes S, Shore R. The role of albumin in developing rodent dental enamel: a possible explanation for white spot hypoplasia. J Dent Res 1992;71:1270-4.
178. Kumar V, Abbas A, Aster J., editors. Robbins Basic Pathology. Philadelphia: Elsevier/Saunders; 2013.
179. Tung K, Fujita H, Yamashita Y, Takagi Y. Effect of turpentine-induced fever during the enamel formation of rat incisor. Arch Oral Biol 2006;51:464-70.
180. Jedeon K, De la Dure-Molla M, Brookes SJ, Loiodice S, Marciano C, Kirkham J, et al. Enamel defects reflect perinatal exposure to bisphenol A. The American journal of pathology 2013;183:108-18.
181. Ryynänen H, Sahlberg C, Lukinmaa P-L, Alaluusua S. The effect of high temperature on the development of mouse dental enamel in vitro. Arch Oral Biol 2014;59:400-6.
182. Sui W, Boyd C, Wright J. Altered pH regulation during enamel development in the cystic fibrosis mouse incisor. J Dent Res 2003;82:388-92.
183. Werneck R, Mira M, Trevilatto P. A critical review: an overview of genetic influence on dental caries. Oral Dis 2010;16:613-23.
184. Neale MC, Eaves LJ, Kendler KS. The power of the classical twin study to resolve variation in threshold traits. Behav Genet 1994;24:239-58.

185. Plomin R, Daniels D. Why are children in the same family so different from one another? Behav Brain Sci 1987;10:1-16.
186. Turkheimer E, Waldron M. Nonshared environment: a theoretical, methodological, and quantitative review. Psychol Bull 2000;126:78.
187. Wray N, Visscher P. Estimating trait heritability. Nature Education 2008;1:29.
188. Wang X, Shaffer J, Weyant R, Cuenco K, DeSensi R, Crout R, et al. Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 2010;44:277-84.
189. Bretz W, Corby P, Hart T, Costa S, Coelho M, Weyant R, et al. Dental caries and microbial acid production in twins. Caries Res 2005;39:168-72.
190. Bretz W, Corby P, Schork N, Robinson M, Coelho M, Costa S, et al. Longitudinal analysis of heritability for dental caries traits. J Dent Res 2005;84:1047-51.
191. Boraas J, Messer L, Till M. A genetic contribution to dental caries, occlusion, and morphology as demonstrated by twins reared apart. J Dent Res 1988;67:1150-5.
192. Conry JP, Messer LB, Boraas JC, Aeppli DP, Bouchard Jr TJ. Dental caries and treatment characteristics in human twins reared apart. Arch Oral Biol 1993;38:937-43.
193. Teare MD, Barrett JH. Genetic linkage studies. The Lancet 2005;366:1036-44.
194. Vieira A, Marazita M, Goldstein-McHenry T. Genome-wide scan finds suggestive caries loci. J Dent Res 2008;87:435-9.
195. Norrgard K, Schultz J. Using SNP data to examine human phenotypic differences. Nature Education 2008;1:85.
196. Shaffer J, Feingold E, Wang X, Lee M, Tcuenco K, Weeks D, et al. GWAS of dental caries patterns in the permanent dentition. J Dent Res 2013;92:38-44.
197. Shaffer J, Wang X, Feingold E, Lee M, Begum F, Weeks D, et al. Genome-wide association scan for childhood caries implicates novel genes. J Dent Res 2011;90:1457-62.
198. Zeng Z, Shaffer J, Wang X, Feingold E, Weeks D, Lee M, et al. Genome-wide association studies of pit-and-fissure-and smooth-surface caries in permanent dentition. J Dent Res 2013:0022034513481976.
199. Wendell S, Wang X, Brown M, Cooper M, DeSensi R, Weyant R, et al. Taste genes associated with dental caries. J Dent Res 2010;89:1198-202.
200. Slayton R, Cooper M, Marazita M. Tuftelin, mutans streptococci, and dental caries susceptibility. J Dent Res 2005;84:711-4.
201. Foley DL, Craig JM, Morley R, Olsson CJ, Dwyer T, Smith K, et al. Prospects for epigenetic epidemiology. Am J Epidemiol 2009;169:389-400.
202. Ollikainen M, Smith KR, Joo EJ-H, Ng HK, Andronikos R, Novakovic B, et al. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. Hum Mol Genet 2010;19:4176-88.
203. Gordon L, Joo JE, Powell JE, Ollikainen M, Novakovic B, Li X, et al. Neonatal DNA methylation profile in human twins is specified by a complex interplay between intrauterine environmental and genetic factors, subject to tissue-specific influence. Genome Res 2012;22:1395-406.
204. Townsend GC, Richards L, Hughes T, Pinkerton S, Schwerdt W. Epigenetic influences may explain dental differences in monozygotic twin pairs. Aust Dent J 2005;50:95-100.

205. Wang J, Sun K, Shen Y, Xu Y, Xie J, Huang R, et al. DNA methylation is critical for tooth agenesis: implications for sporadic non-syndromic anodontia and hypodontia. Sci Rep 2016;6:19162.
206. Kühnisch J, Thiering E, Heitmüller D, Tiesler CM, Grallert H, Heinrich-Weltzien R, et al. Genome-wide association study (GWAS) for molar–incisor hypomineralization (MIH). Clin Oral Investig 2014;18:677-82.
207. Jeremias F, Koruyucu M, Küchler EC, Bayram M, Tuna EB, Deeley K, et al. Genes expressed in dental enamel development are associated with molar-incisor hypomineralization. Arch Oral Biol 2013;58:1434-42.